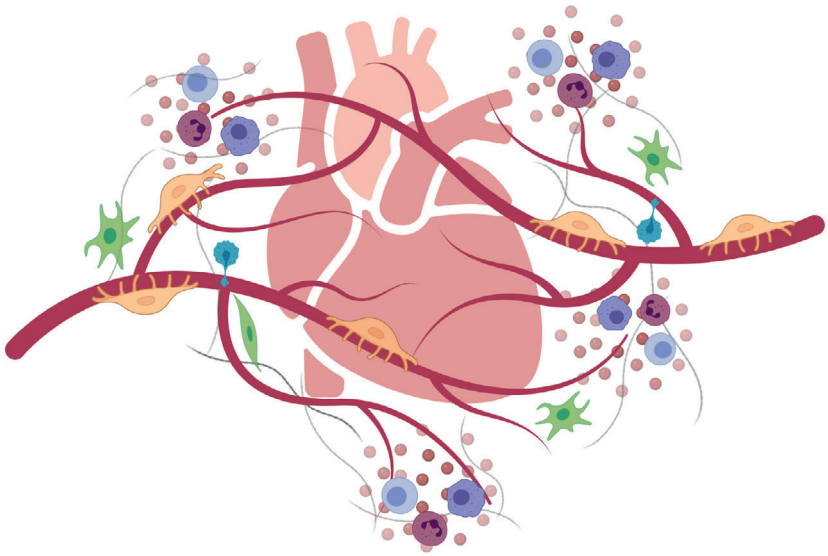
DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

KARTHIK AMUDHALA HEMANTHAKUMAR

Cardiac Endothelial Cells: Potential Therapeutic Targets in Heart Disease



WIHURI RESEARCH INSTITUTE
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"Dedicated to my dear mentors, and to honor the memory of my beloved father who envisioned and aspired this day"

Innovation is the child of freedom and the parent of prosperity

One liner, inspired from the podcasting of the book, "How innovation works" written by Matt Ridley and in discussion with Naval Ravikant.

TABLE OF CONTENTS

ABBREVIATIONS

AAV9	Adeno associated viral vector 9
Angpt1	Angiopoietin 1
Angpt2	Angiopoietin 2
ANP	Atrial natriuretic peptide
BNP	B-type natriuretic peptide
CMCs	Cardiomyocytes
CVD	Cardiovascular diseases
EC	Endothelial cells
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial to mesenchymal transition
EndMT	Endothelial to mesenchymal transition
eNOS	Endothelial nitric oxide synthase
FACS	Fluorescence activated cell sorting
FGF	Fibroblast growth factor
Fhl3	Four and A Half LIM Domains 3
Flk1	Fetal liver kinase 1
Flt1	Fms like tyrosine kinase 1
Flt4	Fms Related Receptor Tyrosine Kinase 4
HB-EGF	Heparin-binding EGF-like growth factor
HGF	Hepatocyte growth factor
IGF1	Insulin growth factor 1
KDR	Kinase insert domain
LV	Lenti viral vector
Mest	Mesoderm specific transcript
NO	Nitric oxide
Nrg1	Neuregulin 1
NRP1	Neuropilin 1
OECD	Organisation for Economic Co-operation and Development
PlGF	Placental growth factor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SASP	Senescence-associated secretory phenotype

SDF1	Stromal cell-derived factor 1
SerpinH1	Serpin Family H Member 1
SMCs	Smooth muscle cells
TAC	Transverse aortic constriction
TGF-B	Transforming growth factor beta
VEGF	Vascular endothelial growth factor
VEGF-B	Vascular endothelial growth factor B
VEGF-C	Vascular endothelial growth factor C
VEGF-D	Vascular endothelial growth factor D
VEGFR1	Vascular endothelial growth factor receptor 1
VEGFR2	Vascular endothelial growth factor receptor 2
VEGFR3	Vascular endothelial growth factor receptor 3
Vwa1	Von Willebrand Factor A Domain Containing 1
WHO	World health organisation

LIST OF ORIGINAL PUBLICATIONS

The thesis is compiled based on the following original research articles; they are cited with in the thesis text by their respective roman numerals.

I. Kivela R*, **Hemanthakumar KA***, Vaparanta K, Robciuc M, Izumiya Y, Kidoya H, Takakura N, Peng X, Sawyer DB, Elenius K, Walsh K and Alitalo K. Endothelial Cells Regulate Physiological Cardiomyocyte Growth via VEGFR2-Mediated Paracrine Signalling. ***Circulation*** *139*, 2570-2584 (2019).

II. Rasanen M, Sultan I*, Paech J*, **Hemanthakumar KA***, Yu W, He L, Tang J, Sun Y, Hlushchuk R, Huan X, Armstrong E, Khoma OZ, Mervaala E, Djonov V, Betsholtz C†, Zhou B†, Kivela R† and Alitalo K. VEGF-B Promotes Endocardium-Derived Coronary Vessel Development and Cardiac Regeneration. ***Circulation*** *143*, 65-77 (2021).

III. **Hemanthakumar KA**, Shentong F, Anisimov A, Mäyränpää M, Mervaala E, Kivela R. Cardiovascular disease risk factors induce mesenchymal features and senescence in mouse cardiac endothelial cells. ***eLife*** *10*, e62678 (2021).

*, † The authors contributed equally.

Publication II was included in the doctoral thesis of Markus Rasanen, University of Helsinki (2019).

ABSTRACT

Cardiovascular diseases (CVD) rank as a number one cause for mortality and accounts for one third of the deaths in several OECD countries (OECD, 2015). According to WHO, CVD pathology is characterised by impaired coronary vasculature associated with cardiac dysfunction, which often results in heart failure (Mendis S, 2011). Although the outlook for prevention and management of CVD risk factors is advancing, the extent of CVD mortality and morbidity remains relatively high (Mendis S, 2011) and the clinical prognosis of heart failure remains poorer than most of the cancers (Braunwald, 2015). Better understanding of the cellular and molecular links between the coronary vasculature and cardiac function under physiological and pathological conditions would enhance the development of personalized targeted therapies for CVD.

In this thesis, my main objective is to define mechanistic insights how proangiogenic cues like VEGF-B or PlGF promote coronary angiogenesis -mediated physiological cardiac hypertrophy and to characterise the effect of CVD risk factors (aging, obesity, physical inactivity and pressure overload) on cardiac endothelial cells (ECs) and cardiac function. We have applied molecular, biochemical, imaging and gene delivery methods to elucidate the phenotypes and molecular mechanisms in in vivo and in vitro model systems.

In the study I, we showed that AAV9-VEGF-B overexpression or endothelial deletion of VEGFR1 increased the bioavailability of the endogenous VEGF to activate VEGFR2 in ECs promoting coronary angiogenesis. Importantly, this indirect activation of VEGFR2 is limited by endogenous levels of VEGF, and it did not promote vascular leakage. VEGFR2 activation induced expression of e.g., *Dll4*, *Notch*, *Apln*, *Apj*, *Klk8* and *Adam12*, indicating activation of Notch and apelin signalling. *Adam12* and *Klk8*, in turn, have been shown to induce shredding of *Hb-egf* and *Nrg1* on the cell surface, leading to activation of *ErbB* receptors present in cardiomyocytes (CMCs). The findings of this study demonstrate a bidirectional crosstalk between ECs and CMCs via VEGFR2, NOTCH and ErbB signalling pathways.

In the study II, overexpression of VEGF-B promoted cardiac EC activation throughout the heart, detected by lineage tracing using AplnCreERT2;Tdtomato reporter mice. However, the VEGF-B -induced EC proliferation was mainly concentrated to the subendocardial myocardium, which was detected by EdU labelling of proliferating cells and staining for a proliferation marker Ki67. In the Study II, my main contribution was the development,

optimization and conduction of cardiac EC isolation for single-cell RNA sequencing. In this study, the main novel finding was that VEGF-B can promote coronary vessel formation from the endocardium during development and after myocardial infarction, which was accompanied by protection from the ischemic insult. In conclusion, the Studies I and II demonstrated the coronary angiogenesis -mediated physiological cardiomyocyte growth is mediated via VEGFR2-NOTCH-ErbB pathways and involves delicate bidirectional crosstalk between ECs and CMC.

In the study III, the effects of CVD risk factors aging, obesity and pressure overload, and exercise training as a physiological stimulus, was studied on cardiac endothelial cells of C57Bl/6J mice. Pure and viable cardiac ECs were isolated and analysed by RNA sequencing and various bioinformatics tools. The data demonstrated that CVD risk factors significantly decreased the number of ECs in the heart as well as the coronary vascular density and cardiac function. Importantly, exercise training improved all of these parameters compared to the sedentary control mice. The next generation RNA sequencing revealed that CVD risk factors significantly remodelled the cardiac EC transcriptome and upregulated several genes and pathways related to inflammation, oxidative stress, TGF-b signalling, vascular permeability, endothelial to mesenchymal transition (EndMT) and cellular senescence, whereas exercise training inhibited most of the same pathways, demonstrating the beneficial role of exercise training on ECs and vasculature. Exercise training also promoted blood vessel development, vascular stability and homeostasis and cell-cell junctions. The gene overlap analysis of the differentially expressed genes in the different data sets revealed SerpinH1 as one of the commonly regulated gene. SerpinH1 was significantly induced by aging and obesity and repressed by exercise training. In vitro studies in human coronary arterial endothelial cells (HCAEC) showed that overexpression of SERPINH1 increased the cell size, induced the expression of EndMT and senescence related transcripts, repressed EC genes and enhanced migration in a wound healing assay. Silencing of SERPINH1 in HCAECs, in turn, completely blocked cell proliferation and decreased collagen deposition and wound healing. As SERPINH1 has previously been linked to fibrosis in other tissues and cell types, and here we found it in all ECs throughout the human and mouse heart, it might be a potential target for the treatment of CVD.

The ECs are not often considered as therapeutic targets, even though in many cases heart problems arise secondary to vascular defects (Heusch et al., 2014). The results from this

thesis suggest that cardiac ECs are highly adaptive to physiological stimuli and maladaptive to pathological stressors. These findings would help to develop innovative and new therapeutic opportunities to treat heart diseases.

REVIEW OF THE LITERATURE

Introduction

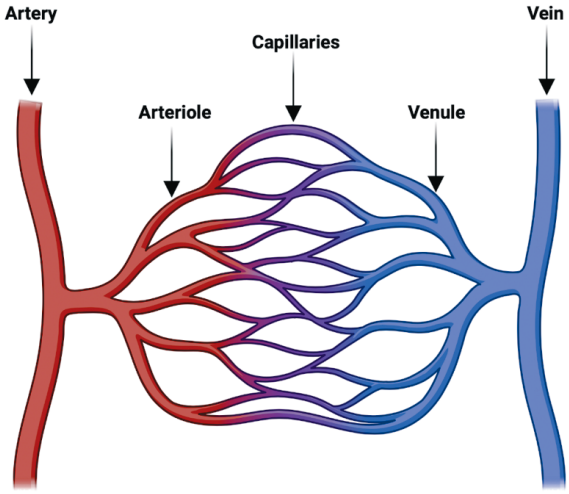
Cardiovascular diseases (CVD) impair coronary vasculature, promote cardiac dysfunction and often result in heart failure, mainly due to behavioural (physical inactivity, unhealthy diet), metabolic (obesity, hypertension, diabetes, cholesterol) and age-related CVD risk factors which accounts for 10% of the global disease burden (Mendis S, 2011).

Endothelial dysfunction occurs in many diseases, but endothelial cells (EC) are not often considered as therapeutic targets, even though in many cases heart problems arise secondary to vascular defects (Heusch et al., 2014). Capillary EC in various organs possess unique structural, functional (secretion of organ specific factors, growth factors, chemokines and adhesion molecules) and phenotypic properties (Nolan et al., 2013; Zhang et al., 2005). The EC are metabolically active, control vasomotor tone, regulate angiogenesis and establish a bidirectional communication between cardiomyocyte (CMC) by paracrine signalling mechanisms (Aird, 2007, 2012; Brutsaert, 2003). Recent findings in the liver and lung have shown that endothelial cells (ECs), rather than being just inert conduits for blood flow, establish an instructive vascular niche by producing paracrine factors (angiocrines), which can stimulate organ growth and regeneration (Rafii et al., 2016).

However, not much is known about the secreted angiocrine factors from the cardiac endothelial cells in response to physiological or pathological stimuli. Understanding the cellular and molecular regulation of ECs in physiological and pathological conditions would provide innovative solutions to develop and sustainably enhance the cardiovascular function in CVD patients.

1. Heart and the cardiovascular system

The primary function of vascular system is to supply oxygen and nutrient rich blood to the tissues and to collect deoxygenated blood and metabolic waste from the tissues. The vascular system is fundamentally classified as hierarchical organization of blood vessels into arteries, arterioles, capillaries, venules and veins (**Figure 1 in the thesis**). The heart acts as a muscular pump within the closed loop circulatory system to pump oxygenated blood and nutrients from the left ventricle for systemic circulation via major arteries branching into smaller arterioles and further to a dense network of capillaries in the target



tissue. The blood capillaries connecting arterioles and venules acts as a major space for the exchange of gases, nutrients, electrolytes and metabolic waste between blood and tissue interstitial fluid. Finally, the deoxygenated blood from the right ventricle enters the lungs for re-oxygenation via pulmonary artery and capillaries in lung alveoli.

Figure 1. Hierarchial organisation of blood vessels.

Cellular diversity and cell communication in the heart

The heart is ellipsoidal in shape, beats approximately 500-600 times/min in mice and 60-100 times/min in humans and the adult heart is composed of four chambers: two upper atrial chambers and two lower ventricular chambers. During cardiogenesis at E10.5, the initiation of septation to partition atrium and ventricles is observed (Xin et al., 2013). The atrial chambers are separated by interatrial septum to form right and left atrium, the interventricular septum further divides the ventricular chambers. Unlike the human heart, the mouse heart does not rest on diaphragm and possess free space to move within the pericardial cavity (Wessels and Sedmera, 2003) and the atrial chambers are considerably small.

The morphology of the heart wall consists of the outermost epicardial layer and the innermost endocardium, which are interconnected by myocardium throughout the atrial and ventricular region (**Figure 2A-B in the thesis**). The heart consists of various cell types (**Figure 2C in the thesis**), primarily classified as myocytes (cardiomyocytes) and non-myocytes (epithelial cells, endothelial cells, smooth muscle cells, mesenchymal cells (pericyte and fibroblasts), pacemaker cells and purkinje fibres, each contributing to the structure and homeostasis of the heart.

In the recent past, the researchers have used advanced qualitative (microscopy) and quantitative (FACS) methods and genetically modified mouse models to reanalyse and revalidate the composition of different cell types in the heart. In adult mouse heart,

endothelial cells (EC) (~42%) are abundantly present, followed by cardiomyocytes (CMC) (~35%), resident mesenchymal cells (fibroblasts and others - ~18%) and leukocytes (~5%) (Pinto et al., 2016; Zhou and Pu, 2016). This data provides basis to uncover the role of individual cell types in heart growth, maintenance, and various heart related diseases.

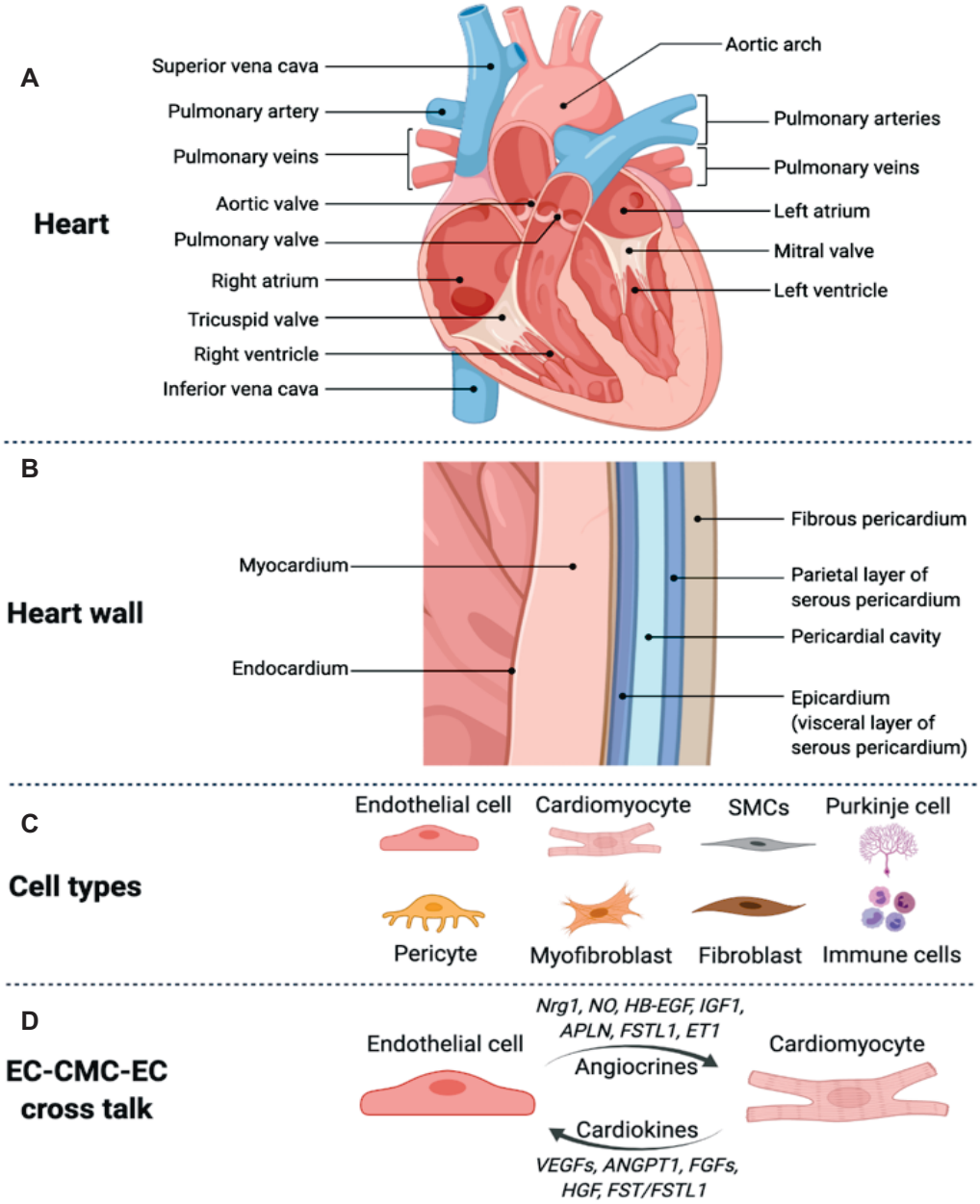


Figure 2. Heart anatomy, cell types and EC-CMC crosstalk. A-B. Longitudinal view of the heart and the heart wall (the anatomical regions are marked in the image), **C.** Different cell types present in the heart, **D.** Intercellular communication between endothelial cells (EC) and cardiomyotes (CMC) and vice versa in the heart.

The different cardiac cell types communicate with each other directly or indirectly via endocrine, autocrine or paracrine factors to maintain the structure, physiological function, and growth of the heart (Brutsaert, 2003; Kamo et al., 2015; Tirziu et al., 2010). The cardiomyocytes in the heart secrete proteins and peptides and these factors are known as cardiokines. For instance, CMC derived factors, like VEGF, induce coronary angiogenesis by activating VEGFR2 signalling in the endothelial cells. Other cardiokines acting on ECs include e.g. VEGFB, PlGF, FGF, Angpt1, HGF and TGFB family members (Talman and Kivela, 2018). The EC derived factors which acts on CMC and other stromal cells via paracrine or juxtracrine signalling are known as angiocrines. These signalling molecules are found to be important for the organ growth, regeneration, homeostasis maintenance, and in tumours they can control tumour progression and metastasis (Ding et al., 2014; Ding et al., 2011; Singhal and Augustin, 2020). Additionally, cardiac cells can also communicate with other cell types via physical contact by forming receptor – ligand complexes (e.g. NOTCH-Dll4), cell adhesion complexes (junction proteins) and cell – extracellular matrix interaction (heparan sulphates, Integrin signalling and other adhesion molecules) (Armingol et al., 2021; Tirziu et al., 2010). Thus, it is evident that intercellular communication is essential to maintain the proper function of the heart, and further understanding these signalling pathways in physiological and pathological conditions are needed to develop new therapeutic approaches (**Figure 2D in the thesis**).

Blood vascular system development

The blood vascular plexus is the primary organ formed during the third week of embryogenesis, serving as a passive conduit for oxygen and nutrients (Saddler and Langman, 2006). The primitive vascular plexus is formed by the differentiation of angioblasts from the mesoderm, and the initial formation of blood vessels from the angioblasts is termed as vasculogenesis (Risau and Flamme, 1995). During later developmental stages and in adults, the new blood vessels are formed from the pre-existing blood vessels by a process known as angiogenesis, either by sprouting or intussusception (Tomanek, 2013).

In mammals, the blood vessels are characterized as larger arteries, smaller arterioles, leading to dense branched network of blood capillaries connecting post capillary venules and veins. In principle, the morphology and composition of the blood vessel wall differs by the size and function of the vessel. The arteries and arterioles structurally resemble the same and consist of three different layers denoted as tunics or tunicae. The tunica intima forms the inner layer of the blood vessel and consists of endothelium and sub-endothelium, the tunica media middle layer of blood vessel is composed of vascular smooth muscle cells and internal elastic lamina, while the external layer tunica externa is composed of collagen, elastin, nerves and fibroblasts. The morphology of the capillaries is different from the pre-capillary blood vessels (arteries and arterioles). The luminal side of the blood capillary wall is lined by a single layer of endothelial cells and basolaterally surrounded by basement membrane and pericytes to maintain the vessel integrity (Tomanek, 2013). In addition to the three different layer of tunics, veins possess valves formed by two leaflets partitioned by extracellular matrix to prevent back flow (Bazigou and Makinen, 2013) (**Figure 1 in the thesis**).

Blood vascular endothelium

The vessel lumen is lined up by a single layer of endothelial cells, which are interconnected primarily by three types of junctions; tight junctions (claudins, occludins, JAM-A, ZO and actin cytoskeleton) mediate ion exchange across endothelium, adherens (cadherins) regulate vascular permeability and gap junctions promote cell-cell signalling (Tomanek, 2013). The structure of the vascular endothelium is organ specific, and they are characterized as continuous, discontinuous and fenestrated. The continuous endothelium is present in the heart, skeletal muscle, lungs, central nervous system, arteries and skin, whereas the discontinuous or sinusoidal vascular endothelium is found in the bone marrow, spleen and liver. The fenestrated endothelium is present in the kidney, intestinal tract, pancreas and endocrine glands. The integration of ECs by junctional proteins plays a major role in vascular integrity, homeostasis and permeability, and they act as barrier for transporting substances across endothelial cells. The ECs drive many metabolic, functional and synthetic functions of the blood vessels. In the heart, the ECs establish a bidirectional communication between cardiomyocytes by paracrine signalling mechanisms (Aird, 2007, 2012).

2. Vascular Endothelial Growth Factors (VEGFs) and their receptors

The VEGFs and VEGF receptors play an indispensable role during embryogenesis and in adults to induce vasculogenesis and blood- and lymphangiogenesis. Until now, five VEGF ligands VEGF/VEGF-A, VEGF-B, VEGF-C, VEGF-D and PLGF have been identified and they bind to their appropriate VEGF receptors VEGFR1/Flt1, VEGFR2/KDR/Flk1 and VEGFR3/Flt4 in mammals (**Figure 3 in the thesis**). The VEGFs are secreted glycoproteins and VEGF receptors are receptor tyrosine kinases (RTKs). The VEGF receptors are activated by binding their respective ligands. Homo- or heterodimeric VEGF receptor complexes mediate their effects by auto or trans-phosphorylation of the tyrosine kinase domains to activate the intracellular downstream targets (Olsson et al., 2006).

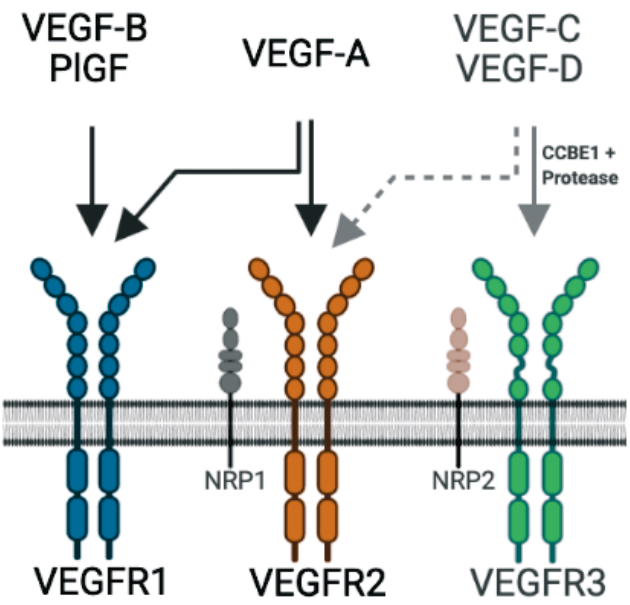


Figure 3. Schematic illustration of different types of vascular endothelial growth factor (VEGF) receptors and their binding partners.

VEGF/VEGF-A

VEGF binds to VEGFR1 and VEGFR2 and promotes blood vessel growth by angiogenesis (Ferrara and Henzel, 1989). It is also known as a vascular permeability factor, as it was first found to regulate vascular permeability and leakage (Senger et al., 1983). VEGF expression is essential during developmental stages, as studies have shown that the mice lacking both Vegf alleles die at E9.5-10.5 due to vascular defects (Carmeliet et al., 1996). Haploinsufficient Vegf mice die due to impaired vascular development around E11-12(Carmeliet et al., 1996; Ferrara et al., 1996). In mouse, three isoforms of Vegf have been identified: VEGF120, VEGF164 and VEGF188. Genetic studies have shown that almost fifty percent of the mice possessing only VEGF120/120 isoform die soon after the birth due to internal bleeding in organs, and the rest die at P14 due to heart failure, aberrant angiogenesis in the myocardium, ischemic cardiomyopathy, skeletal defects, defective vascular outgrowth and patterning in the retina (Carmeliet et al., 1999; Maes et al., 2002;

Stalmans et al., 2002). Mice expressing only VEGF188/188 have defective arterial development in the retina, dwarfism, defective epiphyseal vascularization, impaired development of growth plates and secondary ossification centres, knee-joint dysplasia. In contrast, mice expressing only VEGFA164/164 are healthy, indicating that this is the major isoform (Olsson et al., 2006; Simons et al., 2016). In humans, VEGF undergoes alternative splicing and generates eight different isoforms VEGF121, VEGF145, VEGF148, VEGF162, VEGF165, VEGF183, VEGF189 and VEGF206 (Takahashi and Shibuya, 2005). In addition to endothelial cells, VEGF is found to be expressed in other cell types like fibroblasts, epithelial cells (Pertovaara et al., 1994), macrophages (McLaren et al., 1996), neutrophils (Taichman et al., 1997), hematopoietic stem cells (Bautz et al., 2000), neuronal cells (Haigh et al., 2003), cardiac myofibroblasts (Chintalgattu et al., 2007) and cardiomyocytes (Giordano et al., 2001). In addition to being a paracrine factor, VEGF also induces autocrine signalling in endothelial cells and hematopoietic stem cells (Gerber et al., 2002; Lee et al., 2007). In tumours, elevated VEGF levels initiate pathological angiogenesis leading to increase in tumour size and blood vasculature area (Claesson-Welsh and Welsh, 2013; Kim et al., 1993). VEGF mRNA expression is primarily regulated by hypoxia inducible factors like hypoxia inducible factor 1 (HIF-1a) (Pugh and Ratcliffe, 2003). During embryogenesis and postnatal development stages, VEGF expression is increased and is indispensable for the blood vessel formation and growth, whereas the expression gradually decreases when the mice attain adulthood (Gerber et al., 1999).

VEGF-B

In 1996, Olofsson and colleagues discovered VEGF-B, which was found to share structural similarities with VEGF and PLGF(Olofsson et al., 1996). VEGF-B undergoes alternative splicing to generate two isoforms VEGF-B167 and VEGF-B186. The VEGF-B167 contains heparin-binding domain and binds to heparin sulphate proteoglycans (HSPGs) on the cell surface, whereas VEGF-B186 isoform does not contain heparin-binding domain, which makes it more soluble. Both isoforms bind to membrane bound VEGFR1 and soluble VEGFR1. The proteolytically processed form of VEGF-B186 at Arg-127 can also bind to Neuropilin 1 (NRP1) and the results from previous studies indicate that proteolytic cleavage of VEGF-B186 increases its binding affinity to VEGFR1. Hence, the activation of NRP-1 binding by the proteolytically processed form of VEGF-B186 could enhance the VEGFR1 binding affinity, to promote VEGF-B186 mediated biological effects like governing protease activities in endothelial cells (Makinen et al., 1999; Olofsson et al., 1998). VEGF-B is widely

present in metabolically active tissues, and high mRNA levels were detected especially in the heart, skeletal muscle and adipose tissue (Bry et al., 2014). In the heart, one strain of the VEGF-B knock-out mice have atrio-ventricular conduction abnormality characterized by prolonged PQ interval (Aase et al., 2001) and the other strain showed slightly decreased heart size during development (Bellomo et al., 2000). However, in a subsequent analysis these phenotypes were not confirmed (Dijkstra et al., 2014), indicating very mild or no physiological phenotype in VEGF-B deficient mice. In contrast, several studies have showed effects of VEGF-B overexpression mainly in the heart, but also in adipose tissue and in nervous system. The cardiac-specific transgenic overexpression of full-length VEGF-B induced expansion of coronary vasculature, enlargement of myocardial capillaries, mild cardiomyocyte hypertrophy, and it promoted in vivo ischemia protection(Bry et al., 2010; Karpanen et al., 2008; Kivela et al., 2014). Intramyocardial administration of adeno-viral vector -mediated overexpression of VEGF-B167A in the rat heart prevented the progression of angiotensin II associated left ventricular dysfunction (Serpi et al., 2011). In the rat heart, AAV2 encoding VEGF-B167 increased cardiomyocyte size, promoted antiapoptotic effect on cardiomyocytes after myocardial infarction without significant effect on vasculature (Zentilin et al., 2010). In canines, intramyocardial delivery of AAV9-VEGF-B167 have delayed the onset of heart failure by limiting the apoptosis during tachypacing induced cardiomyopathy (Pepe et al., 2010). Further, In dogs with dilated cardiomyopathy (DCM) intracoronary gene delivery of VEGF-B167 seems to provide protective effect by blocking the development of heart failure development from compensated to decompensated stage with significant transgene efficacy, feasibility and tolerability (Paradies et al., 2019; Woitek et al., 2015). In the adult mouse heart, high adenoviral mediated overexpression of VEGF-B186 might increase the risk for ventricular arrhythmia (Lahteenvuo et al., 2020). In addition, in transgenic mice overexpressing VEGF-B alterations in electrophysiological properties were also observed (Naumenko et al., 2017). The administration of AAV9-VEGF-B186 four weeks after the trans-aortic constriction in mice maintained cardiac function by inducing angiogenesis, blocking apoptosis and enhancing cardiomyocyte proliferation (Huusko et al., 2012). The AAV9-mediated VEGF-B gene delivery in adult mouse has also been shown to induce angiogenesis in adipose tissue, and to improve glucose metabolism in obese mice (Robciuc et al., 2016). It also prevented doxorubicin induced cardiotoxicity by protecting the endothelial cells from cell death and maintaining capillary network in the heart (Rasanen et al., 2016). In the heart, Increase in VEGFB levels activates AKT/mTORC1 and ERK1/2 MAPK pathways partly via VEGFR2 (Bry et al., 2014; Kivela et al., 2014).

VEGF-B is also expressed in CNS (Aase et al., 1999) and the loss of VEGF-B in mice has led to deteriorated recovery of cerebral ischemic injury, and the mice showed signs of motor neuron degeneration when breed with a mouse model with amyotrophic lateral sclerosis, SOD1 mutant mice (Bry et al., 2014; Poesen et al., 2008; Sun et al., 2004). Interestingly, VEGF-B triggered neurogenesis in adult mice, the exogenous administration of VEGF-B167 enhanced the neuronal survival, and in preclinical Parkinson's disease model VEGF-B186 administration conferred neuroprotection (Bry et al., 2014; Falk et al., 2011; Falk et al., 2009; Sun et al., 2006).

PlGF

Placental growth factor (PlGF), encoded by PGF gene, belongs to the VEGF ligand family. The mice lacking PlGF are viable and develop normally during embryogenesis, but under pathological conditions like ischemia, inflammation, wound healing and cancer they developed defective blood vessel growth (Carmeliet et al., 2001; Olsson et al., 2006). In humans, PlGF is expressed in four isoforms PlGF1, PlGF2, PlGF3, PlGF4, whereas in mice only one isoform (PlGF2) is expressed (Maglione et al., 1991; Maglione et al., 1993). Initially, PlGF was found to be expressed in human placenta and its expression is also detected in lungs, skeletal muscles and heart (Ribatti, 2008). Both PlGF and VEGF-B have binding affinity for VEGFR1 but their biological functions are different (Bry et al., 2014). In the mouse heart, PlGF is found to induce myocardial angiogenesis and to mediate cardiac hypertrophy via nitric oxide dependent Akt/mTORC1 pathway (Jaba et al., 2013). In another study, PlGF was found to mediate pressure overload -induced cardiac hypertrophy through paracrine signalling between cardiomyocytes, ECs and fibroblasts via capillary growth and fibroblast proliferation (Accornero and Molkentin, 2011; Accornero et al., 2011). Although the effects of VEGF-B and PlGF in the heart seem to be highly similar, it has been shown that PlGF can induce strong phosphorylation of VEGFR1, whereas VEGF-B induces very little if any phosphorylation (Anisimov et al., 2013; Autiero et al., 2003). In skeletal muscle, AAV9-PlGF overexpression has been previously shown to increase evans blue extravasation compared to AAV9-VEGF-B treated mice, which could be due to strong phosphorylation of VEGFR1 induced by PlGF (Anisimov et al., 2013).

VEGF-C and VEGF-D

VEGF-C was the first discovered primary ligand for VEGFR3 and during embryogenesis it is expressed from E8.5 onwards (Kukk et al., 1996). The mice lacking both VEGF-C alleles

die due to the lack of lymphatic vessels and resulting oedema at embryonic day 15.5-17.5, whereas haploinsufficient VEGF-C mice are viable but they develop hypoplasia in their lymphatics leading to lymphedema (Karkkainen et al., 2004). The full-length VEGF-C binds to VEGFR3 and VEGFR3/2 heterodimer, while VEGFR2 can bind only the proteolytically processed form of VEGF-C. VEGF-D also binds to VEGFR3 and is considered as a lymphangiogenic factor. In contrast to VEGF-C, mice lacking VEGF-D are viable and develop normally with slight reduction of lymphatic vessels in the area near to lung bronchioles (Baldwin et al., 2005). Transgenic overexpression of VEGF-D under the K14 promoter resulted in hyperplasia of the lymphatics in the skin and demonstrated the lymphangiogenic activity of VEGF-D (Veikkola et al., 2001). The proteolytically processed form of VEGF-D can also bind and activate VEGFR2 inducing angiogenesis (Nurro et al., 2016), A one year follow up clinical study in patients with refractory angina, adenoviral (Ad) overexpression of VEGF-D improved myocardial perfusion reserve and the Ad-VEGFD gene therapy was found to be safe and well-tolerated (Hartikainen et al., 2017). Recently, the efficacy and safety of the clinical grade human VEGF-D containing residual replication competent adenovirus were also determined, and the results indicate that the presence of minimal levels of replication competent adenoviral gene therapy may not impose major safety problems (Leikas et al., 2021).

VEGFR1/Flt1

Vascular Endothelial Growth Factor Receptor 1 (VEGFR1), also known as Flt1, consists of a ligand binding domain on the cell surface and intracellular signal transducing tyrosine kinase domain, interconnected by the membrane spanning transmembrane domain (Shibuya, 2013). As a receptor, VEGFR1 binds VEGF, VEGF-B and PlGF (Olsson et al., 2006). Embryonic deletion of VEGFR1 is lethal and the mice die at E8.5-9.0 due to hyperproliferation of endothelial cells resulting in the formation of disorganized vasculature (Fong et al., 1995; Fong et al., 1999). However, the lack of VEGFR1 tyrosine kinase domain does not interfere with physiological development in mice, but in pathological context the lack of TK domain delayed tumour growth (Hiratsuka et al., 2001; Hiratsuka et al., 1998), whereas the deletion of both transmembrane and tyrosine kinase domains resulted in the death of 50% of mice during embryogenesis due to impaired vasculature (Hiratsuka et al., 2005). In adult mice, conditional deletion of VEGFR1 leads to increase in blood capillary density due to the proliferation of endothelial cells in various organs like heart, lungs, liver, kidney and brain, and this protected the heart against myocardial infarction (Ho et al., 2012).

VEGFR1 is highly expressed in endothelial cells, and it is also found in macrophages, monocytes and neuronal cells. Compared to ECs, very low levels of VEGFR1 mRNA were detected in cultured postnatal cardiomyocytes from the rat heart (Zentilin et al., 2010). The soluble VEGFR1 contains only the extracellular ligand-binding domain, which preferably binds VEGF and regulates its bioavailability (Kendall and Thomas, 1993). These various findings on VEGFR1 suggest that VEGFR1 acts as a decoy receptor for VEGF, and the decoy property of the receptor is essential during developmental stages and angiogenesis (Meyer et al., 2006). To maintain vasculature growth in a functional level, proangiogenic feedback signaling mechanisms are essential, the loss of VEGFR1 in post-natal retina triggered changes in the EC sprouting and filopodia extension phenotypes resulted in hyper branching of vasculature without inducing changes in the retinal astrocyte structure (Chappell et al., 2019). Interestingly, in ECs researchers have shown the involvement of membrane bound VEGFR1 spatially drives the anastomosis of blood vessels, which is achieved by ECs with decreased VEGFR1 expression attracts the incoming sprouts to establish a stable connection, these results suggest sprout anastomosis factors are controlled by VEGFA signaling and connections between the vessels are spatially orchestrated by VEGFR1(Nesmith et al., 2017). Further, recent mechanistic studies have shown that the reduced levels of palmitoylation trafficking enzymes like Rab27a affects the palmitoylation of membrane bound VEGFR1 by decreasing the receptor stability and in turn stimulates the sprouting of blood vasculature and angiogenesis (Boucher et al., 2017).

VEGFR2/Flk1/KDR

VEGFR2 is expressed in endothelial cells and indispensable for vasculogenesis and angiogenesis both during embryogenesis and adulthood. The protein is also known as KDR (kinase insert domain) or Flk1 (Fetal liver kinase 1) in human and mouse, respectively. Mice lacking VEGFR2 die during embryogenesis (E8.5) due to impaired blood island formation and vasculogenesis (Shalaby et al., 1995). The binding of VEGF to the 2 and 3 extracellular immunoglobulin like domains of VEGFR2 activates the receptor and induces angiogenesis, however, the binding affinity of VEGF to VEGFR2 is relatively low when compared to its binding affinity to VEGFR1(Fuh et al., 1998; Shinkai et al., 1998). VEGFR2 can also bind proteolytically processed forms of VEGF-C and VEGF-D (McColl et al., 2003). The soluble form of VEGFR2 (sVEGFR2) can be detected in plasma of human and mouse, and it is formed by splicing of the full length VEGFR2 (Ebos et al., 2004). The sVEGFR2 acts as a trap for excess VEGF and VEGF-C, and thereby limits the proliferation of blood and

lymphatic endothelial cells (Albuquerque et al., 2009). VEGFR2 is highly expressed in the endothelial cells of the vasculature during vasculogenesis and in physiological and pathological angiogenesis. In the fully formed vasculature VEGFR2 activity in endothelial cells is significantly reduced (Koch and Claesson-Welsh, 2012), but the baseline expression is preserved to maintain the homeostasis of endothelial cells via autocrine VEGF/VEGFR2 signalling (Lee et al., 2007). VEGF/VEGFR2 axis plays a central role in the proliferation, differentiation and migration of endothelial cells (Koch and Claesson-Welsh, 2012). The VEGFR2 mediated activation of intracellular messenger molecules like Erk1/2 promotes EC migration, proliferation and required to maintain cell homeostasis. Further, the PI3K-AKT pathway participate in several biological process like cell survival, growth, proliferation and apoptosis (Olsson et al., 2006; Simons et al., 2016).

VEGFR3/Flt4

VEGF-C/VEGFR3 signalling plays an important role in the development of lymphatic vessels, it promotes lymphangiogenesis in adult tissues, and their absence leads to lymphedema (Tammela and Alitalo, 2010). VEGFR3 is also expressed in some of the endothelial cells of the blood vasculature, and its expression is increased in angiogenic vasculature of tumours and endothelial tip cells of the developing retinas (Tammela et al., 2008). VEGFR3 can heterodimerise with VEGFR2 (Koch and Claesson-Welsh, 2012). Apart from endothelial cells, VEGFR3 is expressed in osteoblasts, neuronal progenitor cells and macrophages (Le Bras et al., 2006; Orlandini et al., 2006; Schmeisser et al., 2006). The mouse lacking VEGFR3 during embryogenesis die due to impaired vasculature and remodelling of the heart at E10-11(Dumont et al., 1998). The more recent studies showed that VEGFR3 needs VEGFR2 to induce postnatal retinal angiogenesis in mice (Zarkada et al., 2015) and it also plays important function in inducing survival and proliferation signals in endothelial cells (Secker and Harvey, 2015).

Neuropilins

Two Neuropilin receptors (NRPs) have been identified; NRP1 and NRP2. They both have cytoplasmic signalling domain, they bind class III semaphorins to promote neuronal axon guidance repulsive signals and they also act as co-receptors for VEGFRs (Chen et al., 1997; Kolodkin et al., 1997). Mice lacking both NRP1 and NRP2 die during embryonic day E9.5 due to abnormal vascular development (Takashima et al., 2002). Overexpression of NRP1 resulted in various cardiovascular defects, excess blood vessel formation, dilated blood

vessels and anomalies in nervous system and in limbs (Kitsukawa et al., 1995). VEGF family members bind to NRP1 and 2 with different binding affinity. NRP1 binds VEGF-B and PlGF, and its expression is detected in the endocardium and myocardium of the E12.5 embryos and in coronary vessels, myocardial capillaries and epicardial vessels in adult mice (Bry et al., 2014). In ECs, NRP2 promotes cell survival and migration (Favier et al., 2006), induces angiogenesis and lymphangiogenesis (Harman et al., 2020; Karpanen et al., 2006; Xu et al., 2010) (Harman et al., 2020) and it is also involved in EndMT (Grandclement et al., 2011).

3. Angiogenesis and angiocrines in cardiac hypertrophy

Cardiac hypertrophy is referred as an increase in heart size and weight, primarily induced by physiological or pathological stimulus. Physiological hypertrophy is reversible, induced by growth factors or hormones (e.g., IGF1, insulin and thyroid hormone), and activated during postnatal growth, pregnancy or exercise training, which promotes normal or enhanced cardiac function and metabolic activity. On the other hand, pathological hypertrophy is developed by cardiac overload due to e.g. hypertension, myocardial infarction or genetic factors. It is characterized by impaired contractile function, fibrosis, apoptosis, upregulation of fetal gene expression like ANP, BNP and α-Skeletal actin, and it accounts for heart failure related mortalities (Bernardo et al., 2010; Maillet et al., 2013; van Berlo et al., 2013).

The morphology of the hypertrophied heart in various animal models and in human patients suggests that under physiological conditions capillary microvasculature and myocytes grow proportionally, whereas in pathological context the disproportional growth of microvasculature and myocytes leads to capillary rarefaction (Kamo et al., 2015). Angiogenesis in the myocardium is induced by hypoxia and predominantly regulated by the release of angiogenic factors like VEGFs, Angpt1, Angpt2 from cardiomyocytes, and activation of their receptors in endothelial cells. Myocardial angiogenesis can also be enhanced by exogenously administering VEGF, VEGF-B, FGF-2 and -5, SDF1, HGF using gene therapy vectors (Rissanen and Yla-Herttuala, 2007). The dosing of especially VEGF is challenging, as long-term VEGF administration promotes immature angiogenesis and vascular permeability, results in mice death (Robciuc et al., 2016). So far angiogenic gene therapy has been successful on various preclinical models, but not to the same extent in human studies (Koponen et al., 2021). Thus, more research on suitable growth factors and delivery methods are still needed.

In addition to inducing angiogenesis, transgenic overexpression of VEGF-B, PlGF or a secreted angiogenic factor PR39 have shown to induce angiogenesis-mediated cardiac hypertrophy (Jaba et al., 2013; Kivela et al., 2014; Tirziu et al., 2007). Notably, in the cultured neonatal cardiomyocytes, exogenous administration of PR39 and PlGF failed to promote hypertrophic effect, highlighting that vasculature growth and crosstalk between ECs and CMCs is essential for the induction of myocardial hypertrophy by these growth factors (Accornero et al., 2011; Oka et al., 2014; Tirziu et al., 2007). Further, cardiomyocyte-specific transgenic overexpression of VEGF-B in the rats induced physiological cardiac hypertrophy by significantly increasing the coronary vasculature growth and these changes protected the heart from ischemic damage (Bry et al., 2014). The evidence suggests that angiogenesis may control the growth and size of other adult organs as well (Folkman, 1998).

Angiocrine factors

Capillary ECs in various organs possess unique structural, functional (secretion of organ specific factors, growth and chemokine factors) and phenotypic properties (Nolan et al., 2013; Zhang et al., 2005). Recent findings in the tissues like liver, heart, bone and lung have shown that endothelial cells (ECs), rather than being just inert conduits for blood flow, establish an instructive vascular niche by producing paracrine factors known as angiocrines, which can stimulate organ growth and regeneration (Rafii et al., 2016). In pathological conditions, angiocrines also play an important role during inflammation and cancer (Pasquier et al., 2020). There are several evidence suggesting that angiocrines can regulate cardiac hypertrophy and cardiomyocyte growth. These factors include nitric oxide (NO), ErbB ligands (HB-EGF and Nrg1), Apln, IGF1, FSTL1, BMP4, ADM, TSP1, Midkine (Hemanthakumar and Kivela, 2020). Intriguingly, a recent study suggests that also lymphoangiocrine signals derived from cardiac lymphatic endothelial cells provide cardioprotective effect (Liu et al., 2020). However, at present, not much is known about angiocrine factors derived from the cardiac ECs in response to physiological or pathological stimuli. Identifying and targeting angiocrine signaling could open new avenues to treat several cardiovascular diseases.

4. Endothelial cell homeostasis and cardiac function

Endothelial dysfunction and arterial stiffness are common features of vascular aging. Endothelial dysfunction is termed as the change in endothelial cell physiology characterized by the reduction in the secretion levels of the vasodilator factors like nitric oxide and increase

in EC derived constrictive factors (Golbidi and Laher, 2013), resulting in vasoconstrictive, hypercoagulative, proliferative, and proinflammatory state, in turn augments atherosclerosis (Anderson, 1999). Endothelial dysfunction occurs in many diseases, but endothelial cells (ECs) are not often considered as therapeutic targets, even though in many cases heart problems arise secondary to vascular defects (Zhang et al., 2005). Dysfunctional endothelium likely contributes to more diseases than any other tissue in the body as it affects all organs. Many pathological signals may contribute to EC dysfunction, but the molecular mechanisms in many disease states remains to be elucidated.

Endothelial-to-mesenchymal transition

Endothelial-to-mesenchymal transition (EndMT) is a process where ECs lose endothelial marker expression and morphology and acquire a mesenchymal phenotype via series of molecular events (Chen et al., 2020; Islam et al., 2021; Kovacic et al., 2019), largely recapitulating the better-known phenomenon epithelial-to mesenchymal transition (EMT) (**Figure 4 in the thesis**). This EC plasticity is essential during heart development, when ECs transdifferentiate into mesenchymal cells to form the cardiac valves and this process is physiological (Eisenberg and Markwald, 1995; Li et al., 2018). However, the induction of maladaptive EndMT in various organs such as heart, kidney, intestine, brain and aorta has been previously described, leading to fibrosis and organ failure (Chen et al., 2015; Evrard et al., 2016; Hulshoff et al., 2018; Maddaluno et al., 2013; Rieder et al., 2011; Zeisberg et al., 2008; Zeisberg et al., 2007). The activation of transforming growth factor b (TGFb) signalling pathway has been implicated as a driving force for EndMT (Bischoff, 2019; Bischoff et al., 2016; Cooley et al., 2014; Evrard et al., 2016; James and Rafii, 2014). Several studies have recently suggested that EndMT also contributes to the development of various cardiovascular diseases (Zeisberg et al., 2007), e.g. myocardial infarction, atherosclerosis and cardiac fibrosis, as summarized in recent reviews (Kovacic et al., 2019; Li et al., 2018; Sanchez-Duffhues et al., 2018). EndMT changes the phenotype and function of ECs and affects the crosstalk with parenchymal cells. The EMT is known to be involved in many cancer pathologies and notably tumor metastasis, at present there are cancer drugs to inhibit EMT are under clinical evaluation and moreover some of the FDA approved chemotherapeutic agents are known to provide partial inhibitory effect of EMT e.g. Regorafenib mediated targeting of EMT in colorectal cancer (Kovacic et al., 2019; Peterson and Waterhouse, 2016; Santamaria et al., 2017). Hence, targeting of EndMT may hold

therapeutic hope for treating CVD, but currently there is a lack of understanding of the causal relationships and mechanisms linking EndMT and CVD (Kovacic et al., 2019).

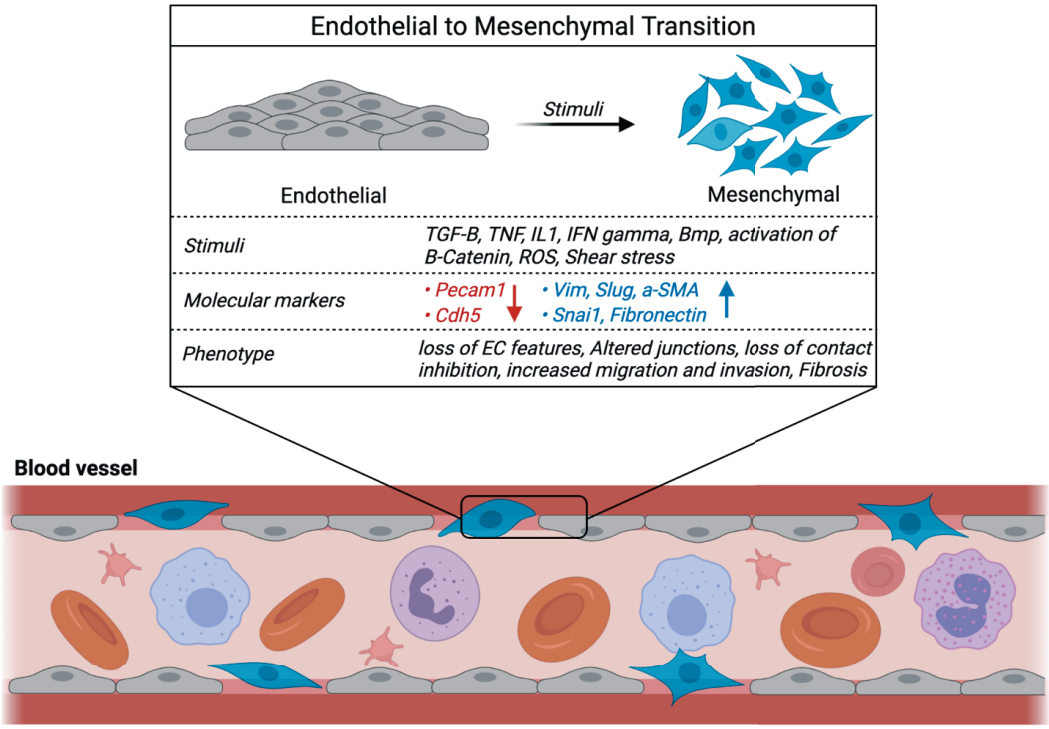


Figure 4. Endothelial to Mesenchymal transition (EndMT) in vasculature. The schematic elucidates the following stimuli which are known to induce EndMT in endothelial cells, expression of endothelial and mesenchymal molecular markers (up- and down- regulated expression are indicated with up and down arrows), the phenotypic features of EndMT cells. In the blood vessel, Endothelial cells and EndMT cells are indicated in grey and blue colour respectively.

Endothelial senescence

Cellular senescence is a phenomenon during which cells stops dividing and acquires phenotypic modifications such as telomere shortening, alterations in secretome profile and tumor suppressor activation (Jia et al., 2019; Rossman et al., 2017). The ECs senescence play a key role in many cardiovascular pathologies and in vascular aging leading to the initiation, progress, and advancement of CVD (Hohensinner et al., 2016). The phenotype of the aged ECs are usually flat and enlarged with multiple nucleus. Further, the changes are accompanied by modulation in cytoskeleton integrity, angiogenesis, proliferation, and cell migration (Uryga and Bennett, 2016). e.g. impaired eNOS productions, increased

endothelin-1 (ET-1) secretion, elevated inflammation and cell apoptosis (Uryga and Bennett, 2016). A large randomized controlled human study PROSPER (Prospective Study of Pravastatin in the Elderly at Risk) suggested that increased levels of endothelial dysfunction markers tPA and Vwf are associated with lower cerebral blood flow in aged adults at high CVD risk (Jia et al., 2019; Sabayan et al., 2014). The outcomes from the ARMYDA-ACS trail (Atorvastatin for Reduction of Myocardial Damage during Angioplasty-Acute Coronary Syndromes) indicates that endothelial progenitor cells may contribute to cardio-protection and reduction of CVD by inducing endogenous vascular repair program. Hence, senescence in ECs promotes inflammation, thrombosis, atherosclerosis, affects vessel tone, angiogenesis, and vascular integrity, and combination of all these results in the development and progression of CVD (Jia et al., 2019). The function of p53 signaling pathway is to maintain genome integrity and homeostasis of the cell to initiate cell survival mechanisms in tumors like cellular senescence, cell cycle arrest, promotes DNA damage repair pathways and induces pathways related to apoptosis to remove damaged cells. However, evidence suggest that to trigger the transcriptional regulation of p53 target genes, the expression levels of p53 expression is crucial. Under high and low stress conditions, p53 elicits different responses, in acute stress conditions p53 will remove the damaged by activating senescence and apoptosis related pathways (Vousden and Prives, 2009; Wu and Prives, 2018). However, the role of p53 in cancer is well appreciated, understanding its role in CVD risk factor pathology may provide a solution to modulate and clear the senescent cells.

5. Cardiovascular diseases risk factors and endothelial cells

Cardiovascular diseases (CVD), which account for approximately 10% of the global disease burden. CVD impair coronary vasculature, promote cardiac dysfunction and often result in heart failure, mainly due to behavioral (physical inactivity, unhealthy diet), metabolic (obesity, hypertension, diabetes, cholesterol) and age-related CVD risk factors (Mendis S, 2011). ECs are adaptive to physiological stimuli and maladaptive to pathological stimuli like oxidative stress, inflammation, senescence. However, it is currently not known how CVD risk factors affect EC transcriptome and phenotype.

Physical activity

Exercise training evokes a whole-body homeostatic program in different cells, tissues and organs, and to compensate this challenge several acute and adaptive responses takes place at molecular, cellular and system level (Hawley et al., 2014). In the heart, the exercise

training has shown to improve the adaptivity of the coronary vasculature by increasing the blood flow reserves and transport reserves, elevated the expression of VEGF and increased the capillary proliferation, vascular density and improved cardiac function (Bloor, 2005; White et al., 1998). Exercise training has shown to provide several beneficial effects not only in healthy individuals, but also in aged individuals (Benjamin et al., 2004; Bhuva et al., 2020), subjects with cardiovascular risk factors and diseases (Hambrecht et al., 1998) and improved the EC function in type 2 diabetic patients (Maiorana et al., 2001). Interestingly, exercise training in adult mice for eight weeks induced cardiomyogenesis in normal and injured hearts (Vujic et al., 2018). Although most of the studies reported so far have demonstrated a beneficial role of exercise training on the heart and cardiac vasculature, future studies understanding the molecular cues driving this feature would help to device new treatment strategies.

Aging

Aging is one of the main CVD risk factors causing ECs dysfunction, which is characterized by the induction of atherogenic and inflammatory gene program, reduced bioavailability of NO, increased release of reactive oxygen species (ROS) and reactive nitrogen species (RNS), decline in the circulating levels of angiogenic factors like VEGF, impaired angiogenesis, poor microvascular perfusion, senescence, apoptosis, perturbed metabolism, deregulation of transport and barrier function of the ECs (Ungvari et al., 2018). The phenotypic and molecular hallmarks of age associated pathologies are genomic instability, attrition of telomere, mitochondrial dysfunction, epigenetic changes, cellular senescence, deregulated cellular cross talk, stem cell depletion, loss of proteostasis and altered nutrient sensing (Lopez-Otin et al., 2013). Uncovering aging at molecular level would help to identify effector and affector signals to develop anti-aging therapies.

AIMS OF THE STUDY

The scope of the study is to investigate the role of cardiac endothelial cells (EC) and angiogenesis during physiological and pathological conditions and to characterize the effects of CVD risk factors on cardiac ECs. The specific objectives of my thesis are as follows:

I. To demonstrate the role of cardiac ECs and angiogenesis in the development of physiological cardiomyocyte growth and to identify the molecular mechanisms by which angiogenesis induces physiological cardiac hypertrophy.

II. To develop and optimize a method to isolate cardiac ECs for single-cell RNA sequencing, to verify the AAV9 tropism in the heart EC and to elucidate the AAV9-VEGF-B mediated endothelial cell activation in the heart using ApInCreERT2; Tdtomato $^{lox/stop/lox}$ lineage tracing mouse model. This was done as a part of a bigger project aimed to determine the potential of VEGF-B to induce formation of blood vessels from the endocardium after myocardial infarction.

III. To characterize the transcriptomic landscape of cardiac ECs in response to different cardiovascular risk factors (aging, physical activity/inactivity, obesity) and in a heart failure model (transverse aortic constriction, TAC). Further, I aimed to identify and validate potential gene targets in ECs for the treatment of cardiovascular diseases.

MATERIALS AND METHODS

Materials

All the mouse lines, cell lines, viral vectors for gene expression or silencing and antibodies used in the three studies of the thesis are listed in the Tables 1-4 below. The detailed description can be found in the original publication I-III.

Table 1. List of mouse lines used in the studies I-III.

Mice strain	Description	Study	Reference
Pdgfb-CreERT2	Conditional Cre expression under *Pdgfb* promoter	I	(Claxton et al., 2008)
Cdh5-CreERT2	Conditional Cre expression under *Cdh5* promoter	I, II	(Okabe et al., 2014)
Myh6-CreERT2	Conditional Cre expression under *Myh6* promoter	I	The Jackson Laboratory #005657
Apln-CreERT2	Conditional Cre expression under *Apln* promoter	II	(Tian et al., 2013)
Npr3-CreERT2	Conditional Cre expression under *Npr3* promoter	II	(Tang et al., 2018)
aMHC-Cre	Constitutive Cre expression under a*MHC* promoter	I	The Jackson Laboratory #011038
aMHC-VEGFB	Overexpression of human VEGFB	II	(Bry et al., 2010)
$Flt1^{lox/lox}$	Gene mutation in the *Flt1* gene	I, II	(Ambati et al., 2006)
$Vegfr2^{e3loxp/e3loxp}$	Gene mutation in the *Vegfr2* gene	I, II	(Hooper et al., 2009)
Vegfr1-TK-/-	Inactivation of gene encoding tyrosine kinase domain of the *Vegfr1* gene	I	(Hiratsuka et al., 1998)
Rosa26-$TdTomato^{lox/STOP/lox}$	Reporter line expressing Td tomato expression after Cre recombination	I, II	The Jackson Laboratory #007914
APJ KO	Apj gene deletion	I	(Ishida et al., 2004)
C57BL/6J	Wild type mice	I, II, III	Envigo Harlan, Janvier labs, Charles river labs

Table 2. Cell lines used in the studies I and III.

Cell line	Description	Study	Source
HCMEC	Human coronary microvascular endothelial cells	I	Promocell
HCAEC	Human coronary arterial endothelial cells	I, III	Promocell
HDMEC	Human dermal microvascular endothelial cell	I	Promocell
HUVEC	Human umbilical venous endothelial cells	I, III	Promocell
bEND.3	Mouse brain endothelial cells	I	ATCC
HCM	Human cardiomyocytes	I	Promocell
HCF	Human cardiac fibroblasts	I	Promocell

Table 3. Viral vectors and recombinant proteins used in the studies I-III.

Vectors/Proteins	Description	Study	Source
AAV9-mVEGF-B186	Adeno associated viral vector encoding mouse VEGFB186	I, II	I,II
AAV9-CTRL	Adeno associated viral vector encoding empty vector	I,II	I,II
AAV9-mPlGF2	Adeno associated viral vector encoding mouse PlGF2	I	I
AAV9-mVEGF-A164	Adeno associated viral vector encoding mouse VEGFA164	I	I
AAV9-mErbb4-ECD	Adeno associated viral vector encoding mouse Erbb4 extracellular domain	I	I
DC101	VEGFR2 inhibitor	I	BioXcell (BE0060)
Afatinib Dimaleate	EGFR inhibitor	I	Selleckchem (S7810)
LV-Scr	Lenti viral vector encoding scrambled plasmid	I, III	I, III
LV-shFlt1	Lenti viral vector encoding short hair pin sequence to silence Flt1 gene	I	I
LV-SERPINH1-Myc	Lenti viral vector encoding overexpression of SERPINH1 gene	III	III
LV-shSERPINH1	Lenti viral vector encoding short hair pin sequence to silence SERPINH1 gene	III	III

Table 4: List of antibodies used in all three studies.

Antibody	Host	Study	Company/Cat. No
CD31	Rat	I, II, III	BD Pharmingen/553370
VEGFR2	Goat	I, II, III	R&D Systems /AF644
αSMA-Cy3	Mouse	I	Sigma/ C6198
Laminin-1	Rabbit	I	Thermo scientific/ Rb-082
Dystrophin	Mouse	I, II	Novocastra/ NCL-DYS2
VEGFR1	Goat	I	R&D Systems / AF471
pVEGFR2	Rabbit	I	Cell signaling/#2478
Hsc70	Mouse	I	Santa Cruz/SC-7298
pEGF Receptor (Y1086)	Rabbit	I	Cell signaling/#2220S
pHER3/ErbB3 (Y1289)	Rabbit	I	Cell signaling/#4791S
pHER4/ErbB4 (Y1284)	Rabbit	I	Cell signaling/#4757S
PI3K-p85	Rabbit	I	Cell signaling/#4292S
pAkt (Ser473)	Rabbit	I	Cell signaling/#9271L
Akt	Rabbit	I	Cell signaling/#9272S
pErk1/2 (T202/Y204)	Rabbit	I	Cell signaling/#9106S
Erk1/2	Rabbit	I	Cell signaling/#9102L
pS6K (Ser235/236)	Rabbit	I	Cell signaling/#2211L
S6K	Mouse	I	Cell signaling/#2317S
Vinculin	Mouse	I	Sigma/#V9131
EGFR (1005)	Rabbit	I	Santa Cruz/SC-03
ErbB3 (C-17)	Rabbit	I	Santa Cruz/SC-285
ErbB4	Rabbit	I	Abcam/ab32375
NRG1- β1	human	I	R&D Systems/MAB3771
HB-EGF	Rabbit	I	Abcam/ab192545
NRG4	Rabbit	I	Biorbyt/b229181
Nrg-1 α/β/1/2 (C-20)	Rabbit	I	Santa Cruz/SC-348
Actin (I-19)	Goat	I	Santa Cruz/SC-1616
RFP	Rabbit	II	Rockland/#600-401-379
Ki67	Rabbit	II	Abcam/ab16667
FITC-CD31	Mouse	II, III	Invitrogen/RM5201

Pacificblue-CD45	Mouse	II, III	Biolegend/103125
Pacificblue-Ter119	Mouse	II, III	Biolegend/116231
PE-Cyanine7- CD140a	Mouse	II, III	eBioscience/25-1401
CD16/CD32 (Fc blocker)	Mouse	II, III	BD Biosciences/553142
VEcadherin	human	III	Cell Signaling/#2500S
Tagln	Sheep	III	R&D Systems/AF7886
c-MYC	Mouse	III	Thermo Fisher/13-2500
HSP47	Mouse	III	Enzo Life Sciences/ADI-SPA- 470-D
Collagen 1	Rabbit	III	Abcam/ab34710
aSMA	Mouse	III	Sigma-Aldrich/A5228
GAPDH	Mouse	III	Sigma-Aldrich/CB1001
DAPI		I, II, III	Sigma-Aldrich/D9542

Methods

All the methods used in the studies I-III are listed in the Table 5 and described below. More detailed descriptions are found in the original publications I-III.

Table 5: Summary of the methods used in the studies.

Methods	Study
In vivo studies	
Adeno-associated viral vector transduction	I, II
Gene deletion in mice	I, II
Blocking of *Vegfr2* and *Egfr/Erbb* signaling	I
Vascular permeability by miles assay	I
Echocardiography	I
Blood pressure measurement	I
Maximal exercise test	I
Treadmill exercise training	III
Transverse aortic constriction	III
In vitro studies	
Cell culture, Lenti viral vector production and transfection	I, III
Wound healing assay	III
Proliferation assay	III
EndMT assay	III
Senescence associated beta galactosidase assay	III
Next generation sequencing of the heart	
Cardiac cell suspension, isolation and analysis of endothelial cells by FACS	II, III
Total RNA sequencing of cardiac endothelial cells	III
Whole genome microarray	I
Molecular, biochemical and statistical analysis	
ELISA	I, II
Real-time quantitative PCR and Western blotting	I, III
Immunofluorescence and immunohistochemical staining	I, II, III
Statistics	I, II, III

In vivo studies

AAV-transduction. The adeno-associated viral vectors (serotype 9) encoding mouse VEGF-B186, PlGF-2, VEGF-A164, ErbB4-ECD and scrambled were generated as previously described (Anisimov et al., 2009) and the constructs were expressed under the CAG promoter. The mice were injected with 2 x 10^{11} AAV9 particles intraperitoneally (i.p.).

Gene deletion in mice. To activate Cre recombination, the adult mice were administered by oral gavage with 2mg per day of tamoxifen dissolved in corn oil for three to five consecutive days and one-week tamoxifen wash out period was adopted.

Blocking VEGFR2 and EGFR/ErbB signaling using receptor tyrosine kinase inhibitors. To inhibit VEGF/VEGFR2 signaling in AAV9-mVEGF-B186/mPlGF2/Ctrl treated C57BL/6J mice, 30 µg/g of DC101 monoclonal antibody (BioXcell) was injected intraperitoneally to the mice every 3-4 days. In the hypertrophy reversal studies, the mice were first injected with AAV's, allowed to express the plasmids and the DC101 antibody treatment was started once the cardiac hypertrophy is established after two weeks. In the prevention studies, the DC101 antibody and AAV's administration started at the same time and lasted for two weeks.

To inhibit EGFR/ErbB signaling in AAV9-mVEGF-B186 transduced hearts, 25 mg/kg of Afatinib Dimaleate (BIBW2992, Cat# S7810, Selleckchem) was administered by oral gavage every day for two weeks and the mice were analyzed.

Vascular Permeability by Miles assay. The mice were anesthetized with ketamine/xylazine, 100µl of 3% Evans Blue dye dissolved in PBS was injected retro-orbitally under anesthesia. The dye was allowed to circulate for one hour and the mice were transcardially perfused with PBS. The heart, lungs, skeletal muscle (tibialis anterior) and kidney were harvested, weighed and Evans blue dye was extracted by incubating the tissues in 500µl of deionized formamide for overnight at 55°C (Heinolainen et al., 2017). Optical Density of the Evans blue was measured at 610 nm. The data is presented as ng of Evans Blue extravasated per mg of tissue dry weight (Radu and Chernoff, 2013).

Echocardiography . The mice were anesthetized by inhalation with 2% isoflurane mixed with 0.5L/min 100% oxygen (Vevo Compact Dual Anesthesia System). Two- dimensional (2D) ECG images were acquired using Vevo 2100 Ultrasound (FUJIFILM VisualSonics).

The images were captured in M-mode along the para- sternal short axis and the modified Simpson's method was applied to measure the left ventricular internal diameter, left ventricular posterior wall thickness, interventricular septum thickness at end- systole and end-diastole. These values were used to calculate left ventricular mass, volume, ejection fraction and fractional shortening using Vevo Vasc Analysis software.

Blood Pressure measurement. The blood pressure (BP) was measured by tail-cuff method (CODA Blood Pressure System, Kent Scientific). In the data acquisition system, the following default values were set for the indicated parameters, Acclimation cycles: 10, Number of sets: 1, Time between sets: 30sec, Cycles per set: 10, Time between cycles: 5sec, Maximum Occlusion Pressure: 250mmHg, Deflation time: 15 sec, Minimum volume: 15µl. The systolic and diastolic BP were recorded.

Maximal exercise test. The exercise capacity of the experimental mice were determined by the incremental maximal running test using a treadmill (LE 8710, Bioseb). The mice were accustomed to the treadmill for two days for 15 min at the speed of 15 m/s. During the maximal exercise test the tread speed was increased from 15 m/s by 5 m/s two times in 5 min interval (for total of 15 min), then the speed was increased by 2 m/s for every 2 min until exhaustion. Total running distance (m) and time (min) were recorded.

Treadmill training program. Mice were trained on a treadmill (LE 8710, Bioseb). The mice were familiarized to the treadmill for three consecutive days with low speed (8-10 cm/s). Progressive training program consisted of 1-1.5 h training bouts five days a week for a total of six weeks with increasing speed, incline and/or duration each week. The following parameters in the treadmill controller were opted, tread inclination: 0°-10°; minimum and maximum tread speed: 10cm to 30cm per second; shock grid intensity: 0.2 mA. The aged mice were exercise-trained for four weeks and the same procedures were followed during the training program.

Transverse aortic constriction. Adult C57BL/6J male mice were anesthetized with ketamine and xylazine. Ligation of the transverse aorta between the right innominate left common carotid arteries against blunted 27-gauge needle with a 7-0 suture was performed and the needle was gently removed. Mice were treated with analgesics (0.05mg/kg of Temgesic i.m.) at the time of the surgery and twice a day for the following two days. For the

control group (sham), all the steps in the surgical procedure were followed, except constricting the aorta. Echocardiography was performed once a week during the experiment.

In vitro studies

Cell culture. Human umbilical vein endothelial cells (HUVEC), human cardiac arterial endothelial cells (HCAEC), human coronary microvascular endothelial cells (HCMEC), human dermal microvascular endothelial cells (HDMEC), mouse brain endothelial cells (bEND.3, ATCC) were purchased from the commercial vendor (Cell lines were authenticated and tested for mycoplasma status by the vendor). The bEND.3 cell line was cultured and maintained in DMEM (Corning, #10-014-CVR) supplemented with 10%FBS, Penicillin (100 U/mL) and streptomycin (100 µg/mL). The rest of the indicated cell lines were cultured and maintained in endothelial cell growth Basal Medium MV (C-22220, PromoCell) supplemented with Supplement Pack GM MV (C- 39220, PromoCell) and gentamycin.

Lentiviral vector production and transfection*.* To clone a lentiviral vector, scrambled sequence in the same vector was used as a control. 293FT cells (ATCC) were cultured and maintained in DMEM supplemented with 10% FCS and L-glutamine, and co-transfected with the lentiviral packaging plasmid vectors CMVg, CMV$_{\Delta}$8.9 and the target plasmid. The supernatants were collected at 48- and 72-hours, and concentrated by ultracentrifugation as described (Lois et al., 2002) . For overexpression, HUVEC and HCAEC were transfected with lentivectors previously for 48 hours. For gene silencing studies, HCAEC were treated with lentivectors encoding for four independent clones of human shSERPINH1 for 24h. Subsequently, the cells were treated with puromycin (2ug/mL) for 48 hours to select the transduced cells. After selection, the cells were used for further analysis. The clone id and target sequence for human shSERPINH1 constructs are shown in the respective article.

Wound healing assay. HCAECs were cultured in the IncuCyte ImageLock 96-well microplate precoated with 0.1% gelatin and cultured in complete EC growth medium. To the cell monolayer, 700 – 800 micron scratch wounds were introduced with IncuCyte WoundMaker. The kinetics of the cell migration were recorded and 10X phase contrast time-lapse images were acquired using IncuCyte Live-Cell Analysis System. The wound closure region was measured by Edge-detection and thresholding method in Image J software (NIH). The data is presented is as wound closure (%) relative to time.

Proliferation assay. The SERPINH1 overexpressed and silenced HCAECs were incubated with 10 μM of EdU labeling solution for 7 hours under normal culture conditions. The Click-iT EdU Alexa Fluor 594 staining kit (Thermo scientific) were used to detect the Edu+ proliferating cells and the nuclei were counterstained with Hoechst. The percentage of Edu+ cells were normalized to Hoechst+ nuclei using area fraction tool (Image J software, NIH). The data is presented as percentage of EdU/Hoechst (%).

EndMT assay. The scrambled or SERPINH1 silenced HCAEC were seeded and cultured in complete EC growth medium. The cells were treated with or without 50ng/ml of recombinant human TGF-β(R&D Technologies) and/or 200μM hydrogen peroxide (Acros organics) for five days as described previously (Evrard et al., 2016; Magenta et al., 2011). The cells were fixed, stained and detected for Tagln+ cells.

Senescence associated beta-galactosidase assay. The SERPINH1 overexpressed HCAECs at passage 6 (P6) were incubated in the fixative solution (#11674, Cell signaling technology) at room temperature for 10 min. The senescence associated beta-galactosidase (SA-β-gal) activity at pH 6.0 was detected with the SA-β-gal staining kit (#9860, Cell Signaling Technology) according to the manufacturer's instructions. The SA-β-gal + cells were quantified using point tool (Image J software, NIH) and normalized to the total number of cells per field. The data presented as percentage of SA-β-gal + cells of all cells.

Next generation sequencing of the heart

Cardiac cell suspension. Mouse heart was dissociated in the 4ml of pre-warmed digestion media containing (1mg/ml of each collagenase types (type I (#17100-017), type II (#17101-015) and type IV (#17104-019) from Gibco were dissolved in DPBS containing 0.3mM $CaCl_2$) and incubated in the water bath at 37°C for 25 min. To neutralize the digestion, 10ml of rinsing media (Dulbecco's modified eagle medium (#31053-028) supplemented with 10% heat inactivated FCS) was added to the cell suspension and filtered through the 70μm nylon cell strainer (Corning, #352350). Throughout the isolation process the cell suspensions were centrifuged for 5min, 300g and 4°C between each rinsing step. The cell pellet was resuspended in 5ml of ice-cold staining buffer (DPBS containing 2% heat inactivated FCS and 1mM EDTA). Before antibody staining, the cells were incubated with Fc receptor blocking antibody (CD16/32) for five minutes. The cells were incubated with the CD31,

PDGFRa/CD140a, CD45, and Ter119 antibodies for 30 min. The cells were rinsed twice with the staining buffer and filtered through 5ml cell strainer tubes (Corning, #352235).

Isolation of Cardiac Endothelial Cells by FACS. Total cells were passed through a 100µm nozzle. The cell populations were gated based on the forward and side- scatter area of the cells (FSC-A and SSC-A). The single cells were selected depending on forward scatter parameters area, height and width of the cells (FSC-A, FSC-H or FSC-W). DAPI was used to determine live and dead cells. To enrich and FACS sort pure and viable cardiac ECs, endothelial cells were stained with CD31, mesenchymal cells with PDGFRa/CD140a, leucocytes with CD45 and red blood cells with Ter119. The live cardiac endothelial cells were defined as CD31 CD45 Ter119 CD140a DAPI. Cells were sorted using FACS Aria II (BD Biosciences), the data was acquired with BD FACSDIVA v8.0.1 and further analyzed with FlowJo v10.1 (FlowJo, LLC) software. We verified the enrichment and purity of the FACS sorted Cardiac EC population (CD31+ PDGFRa (CD140a)- CD45- Ter119- DAPI-) by QPCR analysis for classical cardiac EC markers. Recently, we have used the same isolation method for single-cell RNAseq experiments, and these results show that there is about 3% contamination from other cells types, mainly pericytes and hemangioblasts.

RNA sequencing of cardiac endothelial cells. The RNA was purified using RNeasy Plus Micro Kit (#74034, Qiagen). The RNA integrity was analyzed with bioanalyzer (Agilent Tape Station 4200) and the concentration was determined by Qubit fluorescence assay (ThermoFisher). cDNA libraries were prepared using SMARTer Stranded Total RNA-Seq Kit V2 – Pico Input Mammalian (Takara Bio, USA) kit. The libraries were sequenced using illumina NextSeq 550 System with the following specifications: 1 X 75bp, 50M single end reads were sequenced using NextSeq 500/550 High-Output v2.5 kit. The sequenced reads were analyzed with the following software packages embedded in the Chipster analysis platform 2 (v3.12.2; https://chipster.csc.fi). The DESeq2 Bioconductor package was used to perform the differential gene expression (DGE) analysis. Benjamini-Hochberg correction test to control the false discovery rate, FDR. In our DEG analysis, we have set the FDR (p adj.) cut-off as less than or equal to 0.05 (FDR/p-adj ≤ 0.05) for further downstream pathway analysis and gene overlap analysis.

Whole genome microarray. Total RNA from the heart was analysed using genome-wide Illumina Mouse WG-6 v2 Expression Bead Chips (Illumina), Illumina's Genome Studio

software was used for initial data analysis and quality control and the further data analysis was performed with the Chipster software (www.chipster.csc.fi) (Kallio et al., 2011). After quantile normalization, empirical Bayes method and the Benjamini-Hochberg statistical methods were applied to control the false discovery rate (FDR). The differentially expressed genes with FDR values less than 0.05 (FDR/ P-adj<0.05) were considered significant and used for further analysis.

Molecular and Biochemical studies

ELISA. The serum and tissue levels of the following proteins were determined by ELISA, mVEGF-B186 (in house ELISA was developed using the following antibodies: AF590, BAF767 and recombinant mVEGF-B186 (767-VE from RnD systems), mPlGF (DuoSet DY465, RnD systems), mVEGFR1 (DuoSet DY471, RnD), mVEGFR2 (DuoSet DY493, RnD systems). The Nrg-1 (DuoSet DY377, RnD Systems) kit was used to measure the levels in the conditioned media.

Real time quantitative PCR. Total RNA was purified and isolated using NucleoSpin RNA II Kit (Macherey-Nagel) according to the manufacturer's protocol. iScript cDNA synthesis Kit (Bio-Rad) or High-Capacity cDNA synthesis kit (Applied Biosystems) was used. The gene expression assays were performed using BIO-RAD C1000 thermal cycler. The technical triplicates for each sample were averaged and normalized to the housekeeping genes HPRT-1, TBP or GAPDH and the data presented as fold change. The primer sequences and the TaqMan probe catalog numbers were listed in the respective article.

Immunofluorescence and immunohistochemical staining. Tissue sections or cells on the coverslips were fixed with acetone or 4% paraformaldehyde, blocked with Donkey Immuno Mix (DIM) and incubated with the primary antibodies (Refer to Table 4). Primary antibodies were detected with the corresponding Alexa-conjugated 488, 594 or 647 secondary antibodies (Molecular Probes, Invitrogen). Stained sections were imaged with 40X air or oil immersion objectives using AxioImager fluorescent microscope (Carl Zeiss). The antibody details were listed in the respective article.

Western blotting. RIPA buffer supplemented with aprotinin, 1mg/ml Leupeptin, 1M PMSF, 1M NaF, 0.1M Na3VO4, 0.5% Triton X-100 (v/v) and 0.5% NP-40 (v/v) in PBS pH 7.4 was used to lyse the samples. The total protein concentration was determined using BCA protein

assay kit (Pierce, Thermo Scientific) and resolved in 7.5% Mini-PROTEAN TGX Precast gels (Bio-Rad) and transferred to PVDF membrane (immobilon-P, Millipore). The blots were blocked with 5% BSA (wt/vol) in TBS containing 0.1% Tween 20 and incubated overnight at 4°C with primary antibodies (Table 4). The secondary antibodies were labeled with suitable infrared red fluorescent dye (IR Dye) or HRP-conjugated secondary antibodies (DAKO). The IR dye signals were detected in the suitable fluorescent channels using Odyssey CLx imaging system (Li-COR Biosciences) and the HRP signals were developed with Super-Signal West Pico Chemiluminescent substrate (#34080, Thermo Fisher Scientific) or Femto Maximum Sensitivity Substrate (#34095, Thermo Fisher Scientific) and detected by either Odyssey imager (Li-COR Biosciences) or X-ray films, wherever necessary. The Protein expression in the blots were quantified with Image Studio Lite software (Li-COR Biosciences).

Statistics. The data sets from individual experiments were statistically analyzed by One-way or Two-way ANOVA with Holm-Sidak post hoc test or two-tailed student's t test. $P<0.05$ value was considered statistically significant and P values in the graphs are mentioned as *$P<0.05$, **$P<0.01$ and ***$P<0.001$. The data is represented as mean ± SEM and the GraphPad Prism 7 software was used for statistical analysis.

RESULTS AND DISCUSSION

STUDY I & II. Endothelial VEGFR2 activation is essential for angiogenesis-induced physiological cardiac hypertrophy

The heart structure and function are governed by intercellular communication between heterogeneous population of cells such as endothelial cells, cardiomyocytes, leukocytes and resident mesenchymal cells (Kamo et al., 2015; Tirziu et al., 2010). Endothelial cells in the heart continuously line the coronary vasculature and ventricular chambers promoting the transport of oxygen and nutrients, regulating angiogenesis and engaging in active crosstalk between other cardiac cell types (Aird, 2007, 2012; Brutsaert, 2003; Hemanthakumar and Kivela, 2020; Talman and Kivela, 2018). The results of the studies I and II showed that activation of VEGFR2 either via endothelial VEGFR1 deletion or overexpression of VEGFR1 ligands VEGF-B or PlGF results in cardiac angiogenesis, which promotes mild physiological CMC hypertrophy. Importantly, this indirect VEGFR2 activation does not lead to increased vessel permeability, and it does not induce expression of markers of pathological hypertrophy and fibrosis. Notch and ErbB pathways were found to be activated by VEGFR2 stimulation indicating that these pathways at least partly are responsible for the crosstalk between ECs and CMCs (**Study I**). Furthermore, lineage tracing experiments and proliferation analyses demonstrated that VEGF-B most potently increased proliferation in subendocardial myocardium but activated ECs throughout the heart (**Study II**). As a technological advancement, isolation of a pure and viable population of cardiac ECs for single-cell RNA sequencing was developed and optimized (**Study II**). These findings are discussed in more detail below.

In principle, the healthy growth of the myocardium must be accompanied by expansion of the coronary vasculature to ensure adequate supply of oxygen and nutrients to cardiomyocytes (CMC). In physiological hypertrophy, the heart preserves its oxygen supply by matching the proportional increases in cardiomyocyte size and the extent of coronary microvasculature (Hudlicka et al., 1992; Walsh and Shiojima, 2007), mainly by producing vascular endothelial growth factors (Oka et al., 2014). In pathological state, hypertrophy progression is associated with a mismatch between oxygen supply and demand, as the extent of cardiomyocyte (CMC) hypertrophy is not matched by a corresponding increase in the vasculature (van Berlo et al., 2013). Previous studies have shown that coronary

angiogenesis alone can induce cardiomyocyte growth (Tirziu et al., 2007) and this was further observed in other previous studies. Although, previous studies have shown that the overexpressing angiogenic factors VEGF-B (Bry et al., 2010; Karpanen et al., 2008; Kivela et al., 2014) and PlGF (Accornero and Molkentin, 2011; Jaba et al., 2013). These growth factors induced coordinated expansion of the coronary vasculature, which was accompanied by physiological cardiac hypertrophy. However, based on these studies it was not clear if VEGF-B and PlGF also have direct effects on CMCs in vivo and what are the signaling mechanisms regulating angiogenesis-induced cardiac hypertrophy.

A previous study by Ho et al. demonstrated that global deletion of VEGFR1 in adult mice (using Rosa26-CreERT^{2};Flt1$^{flox/flox}$ mice) resulted in increased VEGFR2 levels in the heart and coronary angiogenesis (Ho et al., 2012). However, the cell specific roles of VEGFR1 and VEGFR2in this process remained unknown. Hence in the study I, the expression of VEGFR1 and VEGFR2 in different human cardiac cell types (Arterial endothelial cells, Micro vascular endothelial cells, Cardiomyocytes and Fibroblasts) was determined. These results indicated that both receptors are highly expressed in the cardiac endothelial cells and very little VEGFR1 expression is detected in the cardiomyocytes (**Study I, Figure 4A and B in the original publication**). This data suggests that the pro-angiogenic factors like VEGF-B and PlGF mainly exert their function via endothelial VEGFR1 and VEGFR2. The receptor expressions were verified using the Tabula Muris data base also in different mouse cardiac cell types at single cell resolution **(Figure 5A-C in this thesis, data not included in the original publication),** showing high expression of both receptors in cardiac endothelial cells and endocardial cells and very little, if any, expression in other cell types.

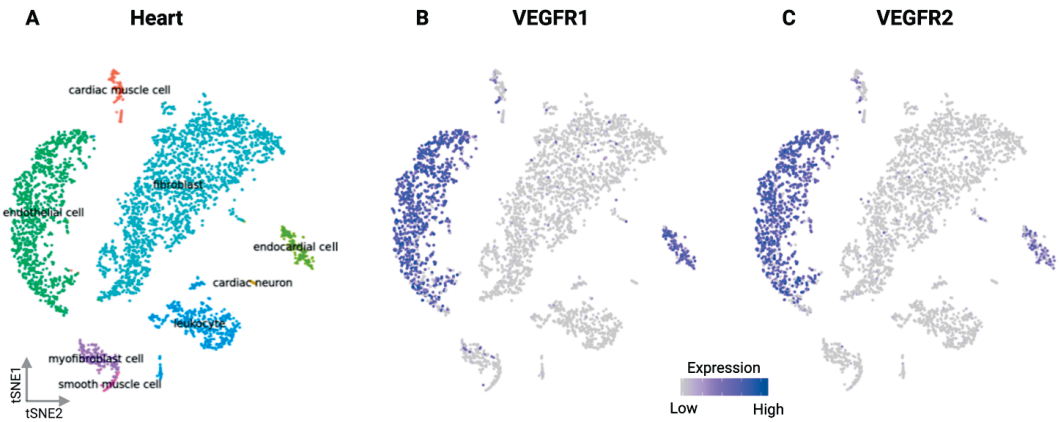


Figure 5. Analysis of VEGFR1 and VEGFR2 expression in various cardiac cell types using tabula muris scRNA sequencing database. A-C. t-SNE plot showing VEGFR1 and VEGFR2 is highly expressed in the cardiac endothelial cells and endocardial cells, but not in other cardiac cell types like fibroblast, cardiac muscle cell, leukocyte, myofibroblast cell, smooth muscle cell and cardiac neuron.

In the study I, conditional deletion of VEGFR1 in endothelial cells (EC) using tamoxifen-inducible PdgfbCreERT2 mouse line and AAV9-mediated overexpression of VEGF-B both induced coronary angiogenesis (approximately increased by 30%) and moderately increased heart-to-body weight ratio (approximately increased by 20%), and the combination of these two treatments even further enhanced the phenotype. The phenotypic characterization of the vasculature and cardiomyocytes in these mice demonstrated proliferation of endothelial cells, increase in coronary vascular density and increase in cardiomyocyte size without increased expression of pathological gene markers (**Study I, Figure 1A-D and Supplemental figure 11C in the original publication**). This was accompanied by lowered blood pressure, probably due to increased vasculature also in peripheral organs, since the VEGFR1 deletion occurs in all endothelial cells and systemic AAV-treatment increases VEGFB levels also in other tissues **(Study I, Supplemental figure 11D in the original publication)**. Further, whole heart transcriptomic analysis of VEGFR1 deletion in PdgfbCreERT2 mice treated with or without AAV9-VEGFB upregulated the expression of NOTCH ligands and receptors and Apln-Apj signaling axis, which both are associated with the CMC growth **(Study I, Figure 2A and 2B in the original publication)**.

Next, miles assay was performed to determine the effect of VEGFR1 deletion in ECs and/or AAV9-VEGF-B overexpression on vascular permeability in the heart, skeletal muscle, kidney and lungs. Intriguingly, neither VEGFR1 deletion in EC or AAV9-VEGF-B overexpression did not induced extravasation of Evans blue, whereas the combination treatment slightly promoted vascular leakage but it was significantly less compared to the mice treated with AAV9-VEGFA164 (**Study I, Supplemental figure 4 in the original publication**). In skeletal muscle, AAV9-PlGF overexpression has been previously shown to increase evans blue extravasation compared to AAV9-VEGF-B treated mice, which could be due to strong phosphorylation of VEGFR1 induced by PlGF (Anisimov et al., 2013). Notably, even long-term VEGFR1 deletion for 9 months in ECs or the combination of VEGFR1 deletion and AAV9-VEGF-B overexpression did not alter ejection fraction and maximal exercise capacity (**Study I, Supplemental figure 11A and B in the original**

publication). These results demonstrate that AAV9-VEGF-B -induced coronary angiogenesis and cardiac hypertrophy are physiological.

In contrast, neither cardiomyocyte-specific deletion of VEGFR1 nor the loss of VEGFR1 tyrosine kinase domain had any effect on heart growth. In addition, the tyrosine kinase domain of VEGFR1 was not needed to mediate the effects of VEGF-B, as the effects of AAV-VEGF-B were similar in VEGFR1 TK-/- and WT mice. Endothelial deletion of VEGFR1 and/or VEGF-B overexpression in cardiomyocytes significantly upregulated VEGFR2 activation in the heart indicating that these treatments prevented VEGF from binding to VEGFR1 and increased the bioavailability of endogenous VEGF towards VEGFR2 as described previously (Sawano et al., 1996; Shibuya, 2013). Indeed, this led to increased VEGFR2 phosphorylation (**Study I, Figure 1H and 1I in the original publication**) in heart lysates. Thus, the results of this thesis indicate that EC-specific deletion of VEGFR1 or VEGFR1-TK deficiency neither inhibited coronary angiogenesis and CMC growth induced by AAV-VEGF-B. Mechanistically, the results showed that both the loss of VEGFR1 in ECs and AAV9-VEGFB overexpression effects were similar and they were mediated by activation of VEGFR2 supporting the previously presented concept of the decoy nature of VEGFR1 (Meyer et al., 2006).

To confirm the role of endothelial VEGFR2 in angiogenesis-induced cardiac hypertrophy, we conditionally deleted VEGFR2 in endothelial cells using Cdh5creERT2 mouse line and overexpressed AAV9-VEGF-B. Additionally, we pharmacologically inhibited the tyrosine kinase activity of the VEGFR2 using DC101 blocking antibody. Both the EC deletion of VEGFR2 and blocking of VEGFR2 tyrosine kinase signalling completely inhibited the VEGF-B -induced coronary angiogenesis and increase in cardiomyocyte size and heart-to-body weight ratio. Next, we tested whether the AAV9-VEGF-B induced cardiac hypertrophy is reversible, a hallmark of physiological cardiac hypertrophy. The C57Bl/6J mice were treated with AAV9-VEGFB for two weeks to develop cardiac hypertrophy and then they were treated with DC101 antibody for additional two weeks. Intriguingly, the treatment fully reversed the heart-to-body weight ratio back to the baseline values, reinforcing the conclusion that AAV9-VEGF-B -induced cardiac hypertrophy resembles physiological hypertrophy. (**Study I, Figure 3A-G, Figure 4D-I in the original publication**).

Our results demonstrated that activation of VEGFR2 promoted the Dll4/NOTCH signalling, which is known to induce coronary angiogenesis and arteriogenesis. Among the most upregulate transcripts were Apln and Apj, ESM1, EfnB2, Klk8 and Adam12 **(Study I, Figure 3G, Figure 5A-C and Supplemental figure 5E in the original publication)**. Adam12 and Klk8 have been shown to mediate shedding of HB-EGF and Nrg1 in other cell types producing soluble cleaved forms of these proteins, which then bind and activate EGFR/ErbB receptors on neighbouring cells.

To investigate the role of EGFR/ErbB signalling in EC-CMC crosstalk, C57Bl/6J mice were injected with AAV9-VEGF-B and treated with afatinib (EGF/ErbB receptor tyrosine kinase inhibitor) or with AAV-vector encoding the extracellular ligand binding domain of the ErbB4 receptor fused with immunoglobulin gamma Fc domain (AAV9-ErbB4-ECD) to inhibit EGFR/ErbB signalling. We then analysed the expression of the downstream effectors of ErbB signalling associated with physiological cardiac growth. The results showed that blocking of the ErbB signalling repressed the expression of transcripts like PI3K-p110B, Akt, Carp/Ankrd1, Tbx3 and signalling messengers like pAkt, pErk1/2, pS6K, which were induced by AAV-VEGF-B (**Study I, Figure A-F in the original publication**). Importantly, blocking the ErbB signalling did not affect AAV9-VEGF-B -induced VEGFR2 expression or coronary angiogenesis. Furthermore, AAV9-VEGF-B overexpression downregulated the expression of C/EBPB, which is downregulated in physiological hypertrophy (Bostrom et al., 2010).

In conclusion, the Study I demonstrated that AAV9-VEGF-B overexpression or endothelial deletion of VEGFR1 promoted coronary angiogenesis and led to physiological cardiac hypertrophy. These results link endothelial VEGFR-Notch signalling to ErbB signalling in cardiomyocytes, revealing one of the mechanisms regulating angiogenesis-induced cardiac hypertrophy and EC-CM interactions. Importantly, AAV9-VEGF-B overexpression did not induce vascular leakage, which often occurs with VEGF administration, thus making it a favourable angiogenic factor to induce physiological angiogenesis. (**Figure 6 in this thesis**).

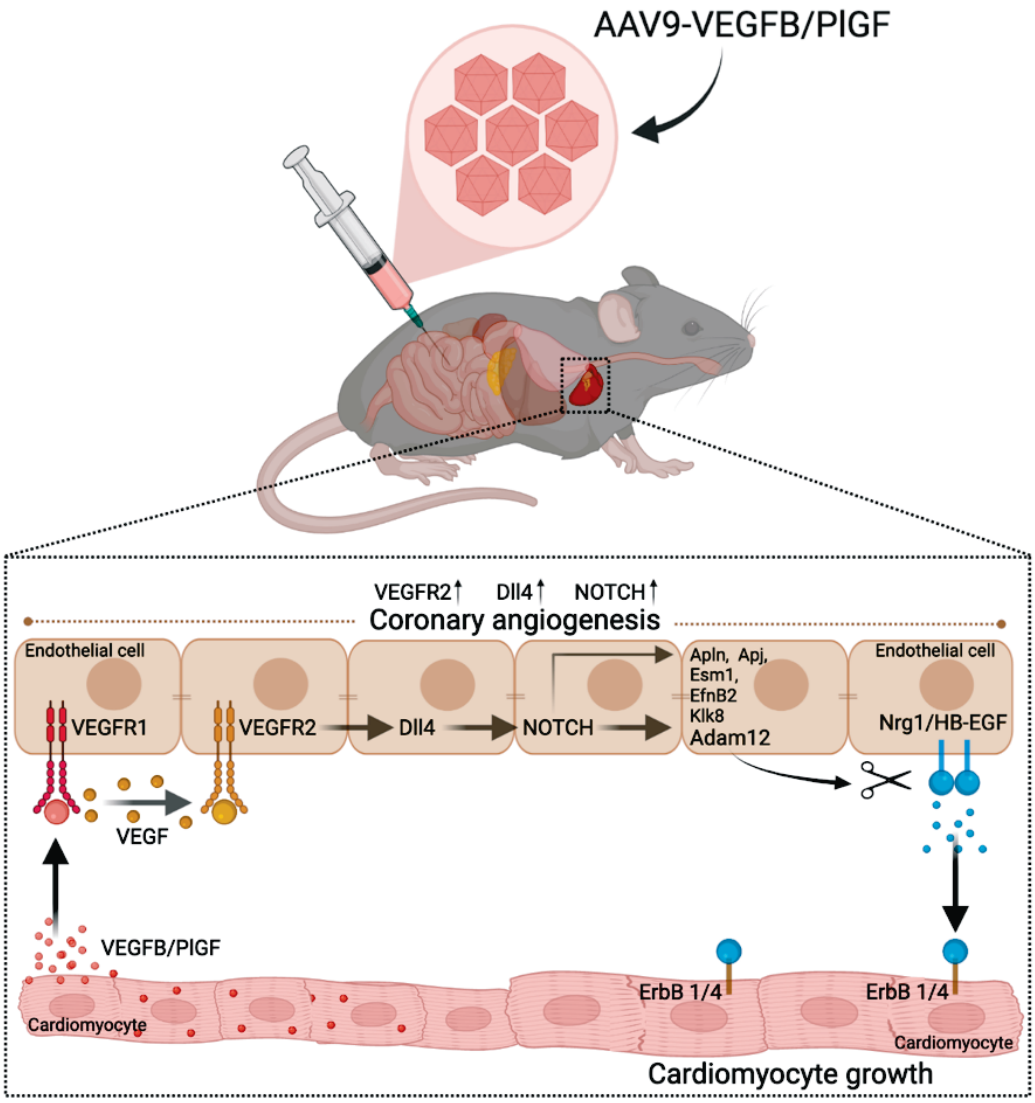


Figure 6. Schematic illustration of VEGFR2 activation in endothelial cells and induction of physiological cardiomyocyte growth mediated by paracrine signalling in the heart. Intraperitoneal (i.p.) administration of AAV9-VEGFB or PlGF in the adult C57Bl/6J mice transduces cardiomyocytes, secretes VEGFB or PlGF, to bind VEGFR1 in the endothelial cells and increases the bioavailability of VEGF and activates VEGFR2 signalling, in turn VEGFR2 activation upregulates the Dll4 and NOTCH expression to promote coronary angiogenesis, additionally activates Apln, Apj, Esm1, EfnB2, Klk8 and Adam12. Adam12 and Klk8 expression cleaves and releases the soluble form of HB-EGF/Nrg1 to bind and activate ErbB receptor 1 and 4 in the cardiomyocyte to induce physiological cardiomyocyte growth.

Since the findings from the Study I showed that AAV-VEGF-B significantly increased the mRNA expression of apelin (Apln), a marker for EC activation, we overexpressed AAV9-VEGF-B in adult AplnCreERT2;TdTomato reporter mouse line to label activated ECs in the heart. Immunofluorescence staining showed increased TdTomato+(Apln+) ECs in coronary vessels located in the epicardium, myocardium and endocardium, suggesting that VEGF-B activates cardiac ECs throughout the heart (**Study II, Supplemental figure 8 in the original publication**). However, proliferation analysis with EdU labeling or Ki67 antibody staining indicated that VEGF-B -induced EC proliferation was most prominent in the subendocardial myocardium (**Study II, Figure 4 and Supplemental figure 6 in the original publication**). This is important as this area is the most vulnerable during ischemia. The most striking finding in this study was that overexpression of VEGF-B was able to induce growth of myocardial vessels from the endocardium either during development or after myocardial infarction. This was accompanied by better cardiac protection in experimental myocardial infarction model (**Study II, Figure 5 and Supplemental figure 9 in the original publication**).

In the study II, my main contribution was to develop, optimize and perform isolation of pure and viable cardiac ECs for single-cell RNA sequencing analyses. The experimental protocol and gating strategies are explained in detail in the original publication (**Study II, Supplemental figure 4 in the original publication, Figure 7 in the thesis)**.

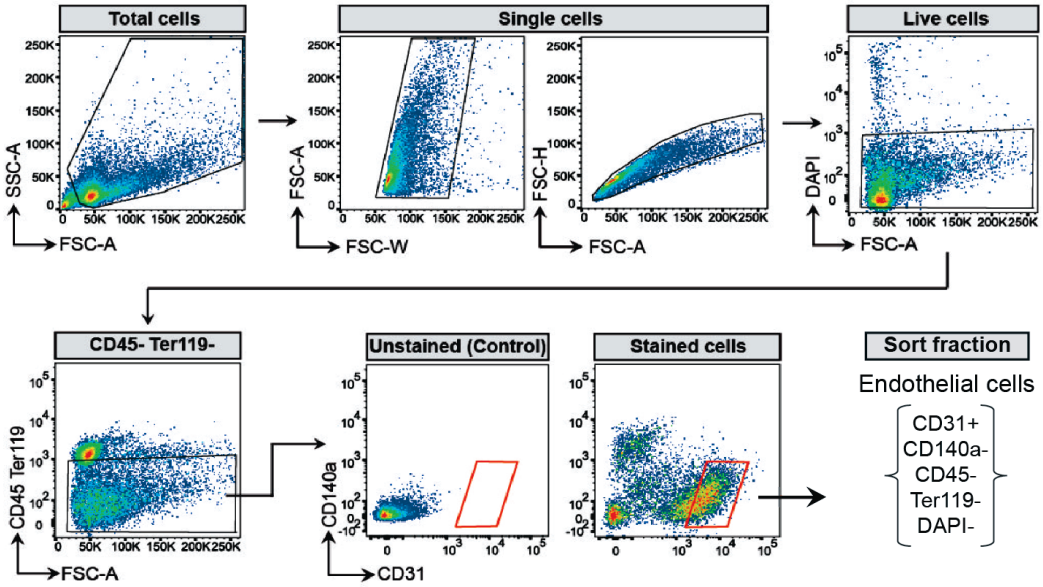


Figure 7. Gating strategy to sort endothelial cells from the heart. The cardiac cell suspension was stained with different cell markers, EC (CD31), mesenchymal cells (CD140a), leucocytes (CD45), red blood cells (Ter119), live and dead cell (DAPI). The stained cardiac total cells were gated based on the forward (FSC-A) and side scatter (SSC-A) parameters of the cells. The single cells were gated, live cells were selected and cells expressing pan-EC marker CD31 and negative for CD140a, CD45 and Ter119 were considered as endothelial cells.

To examine whether AAV9 transduced also endothelial cells in the heart in addition to cardiomyocytes, C57BL/6J mice were injected i.p. with AAV9-vector encoding EGFP or an empty vector for two weeks. Pure and viable cardiac ECs were isolated using FACS. Cardiac EC (CD31+ CD140a- CD45- TER119- DAPI-) population was gated and the EGFP expression was analyzed (**Study II, Supplemental figure 3 in the original publication**). Our data indicate that AAV9 does not transduce ECs, but efficiently transduces CMC as previously shown (Zacchigna et al., 2014).

STUDY III: Cardiovascular disease risk factors trigger mesenchymal gene program and senescence in cardiac endothelial cells

The heart consists of a dense vascular network, and endothelial cells (EC) are highly abundant cell population in the adult heart (Pinto et al., 2016). ECs are found to be highly adaptive to physiological stimuli during normal growth and development (Bloor, 2005; White et al., 1998) and maladaptive to a spectrum of pathological events involving inflammation or oxidative stress (Cines et al., 1998; Gimbrone and Garcia-Cardena, 2016), and the development of heart diseases is often associated with endothelial dysfunction and impaired vascular remodeling. The reports from WHO suggest that ageing, obesity, physical inactivity and hypertension are all highly relevant risk factors for the development of cardiovascular diseases (Mendis S, 2011).

In the study III, I aimed to determine the effect of the CVD risk factors on cardiac ECs. C57Bl/6J wild-type mice were subjected to either physiological stimuli (exercise training) or pathological stimuli (high-fat diet induced obesity (HFD), ageing and transverse aortic constriction -induced heart failure (TAC)). Sedentary lifestyle increases the incidence of several chronic diseases, whereas regular exercise training has positive effects on most of our tissues (Hawley et al., 2014). Although the cardiac benefits of exercise are clear and there have been major advances in unravelling the molecular mechanisms, the understanding of how the molecular effects are linked to the well-being are missing (Hawley et al., 2014), mainly the effect of exercise on cardiac ECs is least studies. In our study, exercise training significantly increased cardiac EC number and vascular density, ejection fraction and also mildly increased the left ventricular mass. In contrast, aging, obesity and TAC resulted in significantly reduced EC number and vascular density, decreased ejection fraction and increase in left ventricular mass. These results showed that the CVD risk factor models used in this study induced capillary rarefaction and deteriorated cardiac function, whereas exercise training had the opposite effects, as was expected.

To decipher the effects of the CVD risk factors on the transcriptomic landscape of cardiac EC, we performed RNA sequencing of the FACS sorted live endothelial cells (CD31+ CD140a- CD45- Ter119- DAPI-). The protocol and results for purity, viability and RNA quality are shown in the **Study III, Figure1-Figure supplement 3A-D in the original publication**. Principal component analysis (PCA) of the EC transcriptome revealed significant proportion

of difference in the gene expression pattern between the experimental groups, and unsupervised hierarchical clustering of EC data sets for all experimental interventions (sedentary, exercise trained, young, aged, sham, TAC) resulted in consistent clustering and significant level of similarity in the gene expression pattern within the groups. Differentially expressed genes (DEGs) analysis showed a large number of up- and downregulated genes especially in aged, obese, and TAC-operated mice followed by a smaller number of affected genes in exercise trained mice, which could be due to the young and healthy control mice, which could move unrestrictedly in their home cages. (**Study III, Figure 2A-J; Figure2-supplement 1A-J; Figure2- supplement 2A-J in the original publication**).

To investigate the biological significance of the DEGs in the CVD risk factor models, we performed Gene ontology (GO) analysis. Comparison of the GO terms suggests that exercise training and the CVD risk factors had strikingly opposite effects on the EC transcriptome. Aging and obesity promoted pathways related to oxidative stress response, activation of inflammatory and fibrosis pathways, cellular aging, TGF-β, SMAD signalling, and they repressed pathways regulating cell number maintenance, tube morphogenesis, vasculature development, EC proliferation and lipid homeostasis. Pressure overload, in turn, activated pathways such as cellular response to TGFBR2 activation of fibrotic pathways, inactivation of cell survival pathways Erk1/2 and MAPK, and ossification process, whereas cellular homeostasis and vasculature development were repressed. Intriguingly, exercise training promoted EC homeostasis, stabilisation, establishment of EC barrier, polarity, focal adhesion and vascular growth, and repressed pathways related to vascular aging, inflammation, permeability, senescence, TGFB1 production, collagen activated tyrosine kinase signalling and mesenchymal gene features. Several pathways upregulated by CVD risk factors were inhibited by exercise training, indicating the cardioprotective role of physical activity by modulating EC phenotype and function (**Study III, Figure 3A; Figure3-supplement 1A-E; Figure3- supplement 2A-E in the original publication**).

The DEGs and the pathway analysis indicated that CVD risk factors upregulated several genes and pathways associated with mesenchymal development, endothelial-to-mesenchymal transition (EndMT), inflammation and cellular senescence. The accumulating evidence suggests that EndMT may play a role in the onset of CVDs (Kovacic et al., 2019; Li et al., 2018; Sanchez-Duffhues et al., 2018; Zeisberg et al., 2007), but currently there is a lack of understanding of the causal relationships and mechanisms linking EndMT and CVD

(Kovacic et al., 2019). The outcome from our analysis increases the evidence and possibility that activation of endothelial TGF-β signalling, and acquisition of mesenchymal features play an important role in the progression of EC maladaptation and heart diseases (Chen et al., 2015; Chen et al., 2019; Kovacic et al., 2019; Xiong et al., 2018). The activation of TGF-β signalling pathway has been implicated to promote EndMT (Bischoff, 2019; Bischoff et al., 2016; Cooley et al., 2014; Evrard et al., 2016; James and Rafii, 2014). Importantly, genes related to TGF-β production and cellular aging were repressed by exercise training, further highlighting the importance of exercise in preventing and delaying the development of CVD.

To study this in more detail, we reviewed our datasets for the expression of endothelial and mesenchymal markers and found significant upregulation of many mesenchymal markers and downregulation of EC genes in aged and obese mice. In two weeks, TAC mice showed upregulation of several mesenchymal markers, whereas after 7 weeks of TAC, there was both up- and downregulation of the EC and mesenchymal markers. This could indicate transient EndMT, similarly to what was recently suggested after myocardial infarction (Tombor et al., 2021). Notably, exercise training downregulated several EndMT genes (*Fscn1*, *Cd93*, *Vwa1*, *Sparc*, *Tuba1a*, *Cd44*, *Trp53*, *Col4a2*, *Mest*, *Cnn2*, *Tnfaip1*, *Lamb1*, *Ltbp4*, and *Unc5b*), the angiogenesis inhibitor gene *Vash1*, and the endothelial activation marker *Apln* and its receptor *Aplnr*. The results were validated using a larger set of samples for *Apln*, *Vim*, *Tgfbr2*, *Vash1*, *Sparc*, and *Tgfb1* genes by qPCR (**Study III, Figure 3B-F; Figure3- supplement 3A-F in the original publication**).

Surprisingly, in addition to the aged mice, we observed a significant upregulation of senescence-associated secretory phenotype (SASP) genes also in the cardiac ECs of HFD and TAC-treated mice. The presence of the SA-B-galactosidase positive cells were confirmed by SA-B-galactosidase staining in the obese hearts. Further studies are needed to characterize the role of cellular senescence and EndMT in cardiac ECs during CVD development.

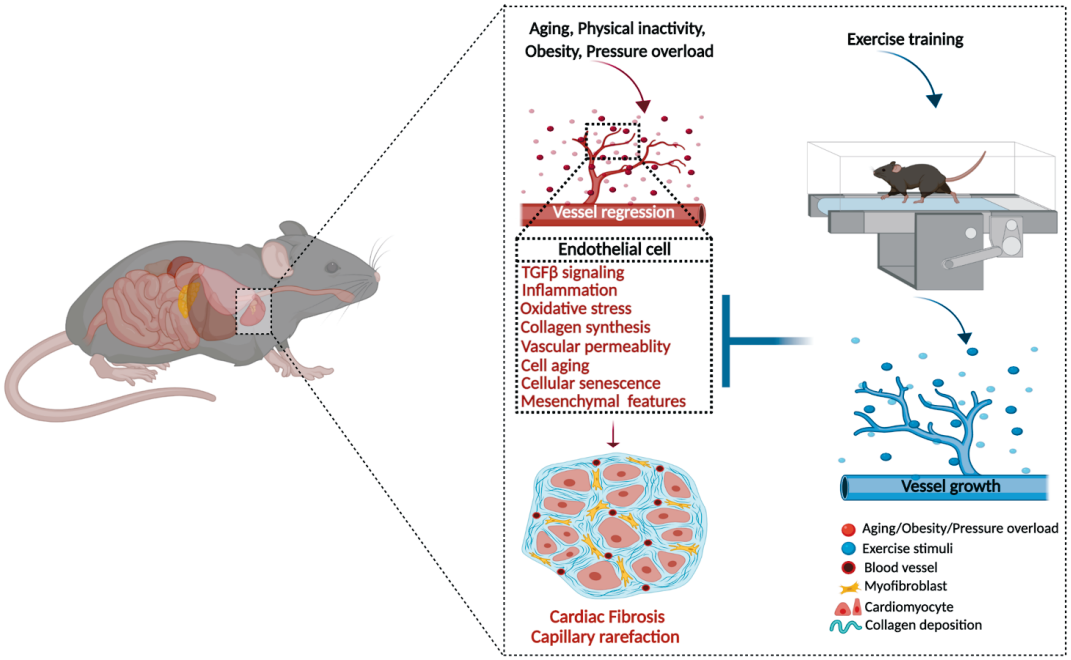


Figure 8. Schematic showing the effect of CVD risk factors (aging, obesity, physical inactivity and Pressure overload) in the endothelial cells of the heart. Aging, obesity, physical inactivity and pressure overload promoted vessel regression in the heart, in the cardiac endothelial activates pathways related to TGFB signalling, inflammation, oxidative stress, cellular senescence and aging, collagen synthesis, vascular permeability and expression of mesenchymal genes leading to cardiac fibrosis and capillary rarefaction, whereas exercise training induced the coronary vascular growth and inhibited many pathways induced by the CVD risk factors.

One of the goals in this study was to identify commonly affected genes in several of the CVD risk factor models. For this, we performed gene overlap analysis of the DEGs from the aged, obese and exercise trained mice. We found four genes, which were significantly affected by all treatments. SerpinH1 and Vwa1 were significantly induced by aging and HFD and repressed by exercise training. The other two genes (Mest and Fhl3) were upregulated by HFD and downregulated by exercise training and aging. Not much is known about Vwa1, thus we focused on Serpinh1, as it has a known role as a collagen chaperone and has been linked to fibrosis in several tissues (Ito and Nagata, 2019). The endothelial expression of *SerpinH1* in CVD risk factor models were validated by qPCR, and at a single-cell level using the Tabula Muris database (Tabula Muris et al., 2018) and cardiac EC atlas (Kalucka et al., 2020). The scRNA sequencing analysis revealed that Serpinh1 is expressed in variety of cell types within the mouse heart, including fibroblasts, myofibroblasts, smooth muscle cells,

ECs, endocardial cells, and to lesser extent in cardiomyocytes (**Study III, Figure 4A-G; Figure4- supplement 1A-D; Figure4- supplement 2A-F; Figure4- supplement 3 in the original publication**). In mouse heart ECs, SerpinH1 was found to be expressed in all clusters, but highly expressed in the apelin-high cluster indicating activated ECs. Additionally, the expression of SERPINH1 is found to be similar in arteries, veins and in various tissues. Interestingly, in the aged mice, exercise training could repress the mRNA expression of the SerpinH1 (**Study III, Figure 4H; Figure3- supplement 1A-E; Figure3- supplement 2A-E in the original publication**).

To understand the role of SERPINH1 in human cardiac ECs, we adopted a gain and loss of function approaches. Overexpression of SERPINH1 using lentiviral vectors (LV-SERPINH1-Myc) resulted in altered cell shape, increased cell size, stress fibre formation and discontinuous endothelial cadherin junctions. Interestingly, overexpression of SERPINH1 promoted mRNA expression of EndMT genes, decreased VE-Cadherin mRNA and protein levels, increased smooth muscle actin -positive cells, slightly increased cell proliferation and enhanced the wound closure in vitro. Notably, overexpression of SerpinH1 in human ECs also induced mRNA expression of senescence associated secretory phenotype genes (SASP) and significantly increased number of SA-B-galactosidase positive cells were detected. Further, to identify the upstream regulators of SERPINH1, we stimulated the cells with TGF-β1 (50ng/ml) and hydrogen peroxide for 5 days, and this stimulus significantly elevated the SERPINH1 expression. Silencing of SERPINH1 in human ECs decreased collagen fibril deposition, completely inhibited cell proliferation and also reduced the expression of Tagln+ cells in TGF-B and/or hydrogen peroxide treated cells. In a recent study, SERPINH1 was found to colocalize with several other EndMT markers in some of the ECs in the left atrium of the patients with atrial fibrillation, a condition often associated to fibrosis (Kato et al., 2017).

In conclusion, this study demonstrated that CVD risk factors significantly remodel the transcriptomic landscape of the cardiac ECs and promote endothelial dysfunction by upregulating the pathways related to endothelial-to-mesenchymal transition, cellular senescence, TGF-β signalling, oxidative stress, vascular permeability, inflammation and collagen synthesis. Importantly, exercise training indicated opposite effects on cardiac EC transcriptome than the CVD risk factors and provided cardiovascular health benefits by

enhancing the coronary vascular growth and improving the cardiac function. Future studies are required to identify a possible therapeutic role of SERPINH1 (**Figure 8 in the thesis**).

CONCLUSIONS AND FUTURE DIRECTIONS

Endothelial cells (EC) line the luminal side of the blood and lymphatic vessels throughout the body, which makes the endothelium as one of the largest and widespread organs. Adult human body is estimated to contain about 1 trillion ECs, which would cover a surface area of approximately 3000 square meters (Jaffe, 1987). As ECs are present in all tissues and that are essential for growth, homeostasis and regeneration, the hypothesis in my thesis was that they are important regulators of cardiomyocyte growth and function and that they are affected by CVD risk factors, which could then contribute to the development of heart diseases. In my thesis, I addressed two major questions: 1) What are the mechanisms mediating the angiogenesis-induced physiological cardiomyocyte growth? and 2) How is the cardiac endothelial cell transcriptome affected by CVD risk factors aging, obesity and pressure overload and what are the effects of exercise training?

In study I and II, we showed that overexpressing VEGF-B using Adeno-associated viral vectors increased the bioavailability of endogenous VEGF resulting in activation of VEGFR2 signalling, which initiated coronary angiogenesis and CMC growth by further activating Notch and ErbB signalling in ECs and CMC, respectively. Notably, coronary angiogenesis induced by VEGF-B overexpression was physiological with about 20-30% increase in vascular area, normal vessel structure and no increase in permeability. In addition, we found that during development and after myocardial infarction, VEGF-B induced vessel growth from endocardium and these vessels seemed to be connected to the ventricle. These findings indicate that instead of VEGF-A, which has not proven optimal growth factor for gene therapy applications because of difficulties in optimal dosing and non-physiological angiogenesis and leakiness of the vessels, VEGF-B could provide a safer alternative. Future studies delineating the EC-CMC interaction in physiological and pathological conditions would help in identifying and validating secreted factors, which participate in maintaining and improving cardiovascular health. Indeed, our and other's findings on the decoy nature of VEGFR1, could provide an interesting avenue to study possibilities of enhancing the activity of endogenous VEGF in spatial and temporal manner to activate endothelial VEGFR2 signalling. This would ameliorate capillary rarefaction and vascular attrition, conditions often seen in cardiovascular disease (CVD) pathologies.

The other part of my thesis aimed to find potential mediators of EC-CMC crosstalk and novel targets for CVD treatment. CVD risk factors had a marked impact on cardiac EC

transcriptome and importantly, exercise training had strikingly similar effect but to the opposite direction. This finding adds to the list of positive effects of exercise on cardiovascular health. We identified SERPINH1 as a new candidate gene mediating the adverse effects of aging and obesity in ECs and this was linked to EndMT and cellular senescence. However, future studies in animal models and in humans will reveal the translational potential of targeting SERPINH1 and EndMT. At least thus far, there are FDA approved chemotherapeutic cancer drugs like regorafenib, which is known to partially inhibit EMT. But, at present there is still a lack of understanding of the causal relationships and mechanisms linking EndMT and CVD. Hence, validating the EndMT phenotype induced by CVD risk factors using lineage tracing mouse models would be advantageous to uncover the therapeutic role of EndMT in treating heart disease. Finally, further mechanistic studies are needed to determine what kind of exercise training could be an optimal intervention to better manage the CVD risk factor -associated micro- and macrovascular complications and other comorbidities by inhibiting e.g., cellular senescence and EndMT.

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REFERENCES

Aase, K., Lymboussaki, A., Kaipainen, A., Olofsson, B., Alitalo, K., and Eriksson, U. (1999). Localization of VEGF-B in the mouse embryo suggests a paracrine role of the growth factor in the developing vasculature. Developmental dynamics : an official publication of the American Association of Anatomists *215*, 12-25.

Aase, K., von Euler, G., Li, X., Ponten, A., Thoren, P., Cao, R., Cao, Y., Olofsson, B., Gebre-Medhin, S., Pekny, M., et al. (2001). Vascular endothelial growth factor-B-deficient mice display an atrial conduction defect. Circulation *104*, 358-364.

Accornero, F., and Molkentin, J.D. (2011). Placental growth factor as a protective paracrine effector in the heart. Trends in cardiovascular medicine *21*, 220-224.

Accornero, F., van Berlo, J.H., Benard, M.J., Lorenz, J.N., Carmeliet, P., and Molkentin, J.D. (2011). Placental growth factor regulates cardiac adaptation and hypertrophy through a paracrine mechanism. Circ Res *109*, 272-280.

Aird, W.C. (2007). Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. Circ Res *100*, 158-173.

Aird, W.C. (2012). Endothelial cell heterogeneity. Cold Spring Harb Perspect Med *2*, a006429.

Albuquerque, R.J., Hayashi, T., Cho, W.G., Kleinman, M.E., Dridi, S., Takeda, A., Baffi, J.Z., Yamada, K., Kaneko, H., Green, M.G., et al. (2009). Alternatively spliced vascular endothelial growth factor receptor-2 is an essential endogenous inhibitor of lymphatic vessel growth. Nat Med *15*, 1023-1030.

Ambati, B.K., Nozaki, M., Singh, N., Takeda, A., Jani, P.D., Suthar, T., Albuquerque, R.J., Richter, E., Sakurai, E., Newcomb, M.T., et al. (2006). Corneal avascularity is due to soluble VEGF receptor-1. Nature *443*, 993-997.

Anderson, T.J. (1999). Assessment and treatment of endothelial dysfunction in humans. J Am Coll Cardiol *34*, 631-638.

Anisimov, A., Alitalo, A., Korpisalo, P., Soronen, J., Kaijalainen, S., Leppanen, V.M., Jeltsch, M., Yla-Herttuala, S., and Alitalo, K. (2009). Activated forms of VEGF-C and VEGF-D provide improved vascular function in skeletal muscle. Circ Res *104*, 1302-1312.

Anisimov, A., Leppanen, V.M., Tvorogov, D., Zarkada, G., Jeltsch, M., Holopainen, T., Kaijalainen, S., and Alitalo, K. (2013). The basis for the distinct biological activities of vascular endothelial growth factor receptor-1 ligands. Sci Signal *6*, ra52.

Armingol, E., Officer, A., Harismendy, O., and Lewis, N.E. (2021). Deciphering cell-cell interactions and communication from gene expression. Nat Rev Genet *22*, 71-88.

Autiero, M., Waltenberger, J., Communi, D., Kranz, A., Moons, L., Lambrechts, D., Kroll, J., Plaisance, S., De Mol, M., Bono, F., et al. (2003). Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. Nature medicine *9*, 936-943.

Baldwin, M.E., Halford, M.M., Roufail, S., Williams, R.A., Hibbs, M.L., Grail, D., Kubo, H., Stacker, S.A., and Achen, M.G. (2005). Vascular endothelial growth factor D is dispensable for development of the lymphatic system. Molecular and cellular biology *25*, 2441-2449.

Bautz, F., Rafii, S., Kanz, L., and Mohle, R. (2000). Expression and secretion of vascular endothelial growth factor-A by cytokine-stimulated hematopoietic progenitor cells. Possible role in the hematopoietic microenvironment. Exp Hematol *28*, 700-706.

Bazigou, E., and Makinen, T. (2013). Flow control in our vessels: vascular valves make sure there is no way back. Cell Mol Life Sci *70*, 1055-1066.

Bellomo, D., Headrick, J.P., Silins, G.U., Paterson, C.A., Thomas, P.S., Gartside, M., Mould, A., Cahill, M.M., Tonks, I.D., Grimmond, S.M., et al. (2000). Mice lacking the vascular endothelial growth factor-B gene (Vegfb) have smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia. Circ Res *86*, E29-35.

Benjamin, E.J., Larson, M.G., Keyes, M.J., Mitchell, G.F., Vasan, R.S., Keaney, J.F., Jr., Lehman, B.T., Fan, S., Osypiuk, E., and Vita, J.A. (2004). Clinical correlates and heritability of flow-mediated dilation in the community: the Framingham Heart Study. Circulation *109*, 613-619.

Bernardo, B.C., Weeks, K.L., Pretorius, L., and McMullen, J.R. (2010). Molecular distinction between physiological and pathological cardiac hypertrophy: experimental findings and therapeutic strategies. Pharmacol Ther *128*, 191-227.

Bhuva, A.N., D'Silva, A., Torlasco, C., Jones, S., Nadarajan, N., Van Zalen, J., Chaturvedi, N., Lloyd, G., Sharma, S., Moon, J.C., et al. (2020). Training for a First-Time Marathon Reverses Age-Related Aortic Stiffening. J Am Coll Cardiol *75*, 60-71.

Bischoff, J. (2019). Endothelial-to-Mesenchymal Transition. Circ Res *124*, 1163-1165.

Bischoff, J., Casanovas, G., Wylie-Sears, J., Kim, D.H., Bartko, P.E., Guerrero, J.L., Dal-Bianco, J.P., Beaudoin, J., Garcia, M.L., Sullivan, S.M., et al. (2016). CD45 Expression in Mitral Valve Endothelial Cells After Myocardial Infarction. Circ Res *119*, 1215-1225.

Bloor, C.M. (2005). Angiogenesis during exercise and training. Angiogenesis *8*, 263-271.

Bostrom, P., Mann, N., Wu, J., Quintero, P.A., Plovie, E.R., Panakova, D., Gupta, R.K., Xiao, C., MacRae, C.A., Rosenzweig, A., et al. (2010). C/EBPbeta controls exercise-induced cardiac growth and protects against pathological cardiac remodeling. Cell *143*, 1072-1083.

Boucher, J.M., Clark, R.P., Chong, D.C., Citrin, K.M., Wylie, L.A., and Bautch, V.L. (2017). Dynamic alterations in decoy VEGF receptor-1 stability regulate angiogenesis. Nat Commun *8*, 15699.

Braunwald, E. (2015). The war against heart failure: the Lancet lecture. Lancet *385*, 812-824.

Brutsaert, D.L. (2003). Cardiac endothelial-myocardial signaling: its role in cardiac growth, contractile performance, and rhythmicity. Physiol Rev *83*, 59-115.

Bry, M., Kivela, R., Holopainen, T., Anisimov, A., Tammela, T., Soronen, J., Silvola, J., Saraste, A., Jeltsch, M., Korpisalo, P., et al. (2010). Vascular endothelial growth factor-B acts as a coronary growth factor in transgenic rats without inducing angiogenesis, vascular leak, or inflammation. Circulation *122*, 1725-1733.

Bry, M., Kivela, R., Leppanen, V.M., and Alitalo, K. (2014). Vascular endothelial growth factor-B in physiology and disease. Physiol Rev *94*, 779-794.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., et al. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature *380*, 435-439.

Carmeliet, P., Moons, L., Luttun, A., Vincenti, V., Compernolle, V., De Mol, M., Wu, Y., Bono, F., Devy, L., Beck, H., et al. (2001). Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med *7*, 575-583.

Carmeliet, P., Ng, Y.S., Nuyens, D., Theilmeier, G., Brusselmans, K., Cornelissen, I., Ehler, E., Kakkar, V.V., Stalmans, I., Mattot, V., et al. (1999). Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. Nat Med *5*, 495-502.

Chappell, J.C., Darden, J., Payne, L.B., Fink, K., and Bautch, V.L. (2019). Blood Vessel Patterning on Retinal Astrocytes Requires Endothelial Flt-1 (VEGFR-1). J Dev Biol *7*.

Chen, H., Chedotal, A., He, Z., Goodman, C.S., and Tessier-Lavigne, M. (1997). Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. Neuron *19*, 547-559.

Chen, P.Y., Qin, L., Baeyens, N., Li, G., Afolabi, T., Budatha, M., Tellides, G., Schwartz, M.A., and Simons, M. (2015). Endothelial-to-mesenchymal transition drives atherosclerosis progression. J Clin Invest *125*, 4514-4528.

Chen, P.Y., Qin, L., Li, G., Wang, Z., Dahlman, J.E., Malagon-Lopez, J., Gujja, S., Cilfone, N.A., Kauffman, K.J., Sun, L., et al. (2019). Endothelial TGF-beta signalling drives vascular inflammation and atherosclerosis. Nat Metab *1*, 912-926.

Chen, P.Y., Schwartz, M.A., and Simons, M. (2020). Endothelial-to-Mesenchymal Transition, Vascular Inflammation, and Atherosclerosis. Front Cardiovasc Med *7*, 53.

Chintalgattu, V., Harris, G.S., Akula, S.M., and Katwa, L.C. (2007). PPAR-gamma agonists induce the expression of VEGF and its receptors in cultured cardiac myofibroblasts. Cardiovasc Res *74*, 140-150.

Cines, D.B., Pollak, E.S., Buck, C.A., Loscalzo, J., Zimmerman, G.A., McEver, R.P., Pober, J.S., Wick, T.M., Konkle, B.A., Schwartz, B.S., et al. (1998). Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood *91*, 3527-3561.

Claesson-Welsh, L., and Welsh, M. (2013). VEGFA and tumour angiogenesis. J Intern Med *273*, 114-127.

Claxton, S., Kostourou, V., Jadeja, S., Chambon, P., Hodivala-Dilke, K., and Fruttiger, M. (2008). Efficient, inducible Cre-recombinase activation in vascular endothelium. Genesis *46*, 74-80.

Cooley, B.C., Nevado, J., Mellad, J., Yang, D., St Hilaire, C., Negro, A., Fang, F., Chen, G., San, H., Walts, A.D., et al. (2014). TGF-beta signaling mediates endothelial-to-mesenchymal transition (EndMT) during vein graft remodeling. Sci Transl Med *6*, 227ra234.

Dijkstra, M.H., Pirinen, E., Huusko, J., Kivela, R., Schenkwein, D., Alitalo, K., and Yla-Herttuala, S. (2014). Lack of cardiac and high-fat diet induced metabolic phenotypes in two independent strains of Vegf-b knockout mice. Scientific reports *4*, 6238.

Ding, B.S., Cao, Z., Lis, R., Nolan, D.J., Guo, P., Simons, M., Penfold, M.E., Shido, K., Rabbany, S.Y., and Rafii, S. (2014). Divergent angiocrine signals from vascular niche balance liver regeneration and fibrosis. Nature *505*, 97-102.

Ding, B.S., Nolan, D.J., Guo, P., Babazadeh, A.O., Cao, Z., Rosenwaks, Z., Crystal, R.G., Simons, M., Sato, T.N., Worgall, S., et al. (2011). Endothelial-derived angiocrine signals induce and sustain regenerative lung alveolarization. Cell *147*, 539-553.

Dumont, D.J., Jussila, L., Taipale, J., Lymboussaki, A., Mustonen, T., Pajusola, K., Breitman, M., and Alitalo, K. (1998). Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. Science *282*, 946-949.

Ebos, J.M., Bocci, G., Man, S., Thorpe, P.E., Hicklin, D.J., Zhou, D., Jia, X., and Kerbel, R.S. (2004). A naturally occurring soluble form of vascular endothelial growth factor receptor 2 detected in mouse and human plasma. Mol Cancer Res *2*, 315-326.

Eisenberg, L.M., and Markwald, R.R. (1995). Molecular regulation of atrioventricular valvuloseptal morphogenesis. Circ Res *77*, 1-6.

Evrard, S.M., Lecce, L., Michelis, K.C., Nomura-Kitabayashi, A., Pandey, G., Purushothaman, K.R., d'Escamard, V., Li, J.R., Hadri, L., Fujitani, K., et al. (2016). Endothelial to mesenchymal transition is common in atherosclerotic lesions and is associated with plaque instability. Nat Commun *7*, 11853.

Falk, T., Yue, X., Zhang, S., McCourt, A.D., Yee, B.J., Gonzalez, R.T., and Sherman, S.J. (2011). Vascular endothelial growth factor-B is neuroprotective in an in vivo rat model of Parkinson's disease. Neurosci Lett *496*, 43-47.

Falk, T., Zhang, S., and Sherman, S.J. (2009). Vascular endothelial growth factor B (VEGF-B) is up-regulated and exogenous VEGF-B is neuroprotective in a culture model of Parkinson's disease. Mol Neurodegener *4*, 49.

Favier, B., Alam, A., Barron, P., Bonnin, J., Laboudie, P., Fons, P., Mandron, M., Herault, J.P., Neufeld, G., Savi, P., et al. (2006). Neuropilin-2 interacts with VEGFR-2 and VEGFR-3 and promotes human endothelial cell survival and migration. Blood *108*, 1243-1250.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K.S., Powell-Braxton, L., Hillan, K.J., and Moore, M.W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature *380*, 439-442.

Ferrara, N., and Henzel, W.J. (1989). Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun *161*, 851-858.

Folkman, J. (1998). Is tissue mass regulated by vascular endothelial cells? Prostate as the first evidence. Endocrinology *139*, 441-442.

Fong, G.H., Rossant, J., Gertsenstein, M., and Breitman, M.L. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature *376*, 66-70.

Fong, G.H., Zhang, L., Bryce, D.M., and Peng, J. (1999). Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. Development *126*, 3015-3025.

Fuh, G., Li, B., Crowley, C., Cunningham, B., and Wells, J.A. (1998). Requirements for binding and signaling of the kinase domain receptor for vascular endothelial growth factor. J Biol Chem *273*, 11197-11204.

Gerber, H.P., Hillan, K.J., Ryan, A.M., Kowalski, J., Keller, G.A., Rangell, L., Wright, B.D., Radtke, F., Aguet, M., and Ferrara, N. (1999). VEGF is required for growth and survival in neonatal mice. Development *126*, 1149-1159.

Gerber, H.P., Malik, A.K., Solar, G.P., Sherman, D., Liang, X.H., Meng, G., Hong, K., Marsters, J.C., and Ferrara, N. (2002). VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism. Nature *417*, 954-958.

Gimbrone, M.A., Jr., and Garcia-Cardena, G. (2016). Endothelial Cell Dysfunction and the Pathobiology of Atherosclerosis. Circ Res *118*, 620-636.

Giordano, F.J., Gerber, H.P., Williams, S.P., VanBruggen, N., Bunting, S., Ruiz-Lozano, P., Gu, Y., Nath, A.K., Huang, Y., Hickey, R., et al. (2001). A cardiac myocyte vascular endothelial growth factor paracrine pathway is required to maintain cardiac function. Proc Natl Acad Sci U S A *98*, 5780-5785.

Golbidi, S., and Laher, I. (2013). Exercise and the aging endothelium. J Diabetes Res *2013*, 789607.

Grandclement, C., Pallandre, J.R., Valmary Degano, S., Viel, E., Bouard, A., Balland, J., Remy-Martin, J.P., Simon, B., Rouleau, A., Boireau, W., et al. (2011). Neuropilin-2 expression promotes TGF-beta1-mediated epithelial to mesenchymal transition in colorectal cancer cells. PLoS One *6*, e20444.

Haigh, J.J., Morelli, P.I., Gerhardt, H., Haigh, K., Tsien, J., Damert, A., Miquerol, L., Muhlner, U., Klein, R., Ferrara, N., et al. (2003). Cortical and retinal defects caused by dosage-dependent reductions in VEGF-A paracrine signaling. Dev Biol *262*, 225-241.

Hambrecht, R., Fiehn, E., Weigl, C., Gielen, S., Hamann, C., Kaiser, R., Yu, J., Adams, V., Niebauer, J., and Schuler, G. (1998). Regular physical exercise corrects endothelial dysfunction and improves exercise capacity in patients with chronic heart failure. Circulation *98*, 2709-2715.

Harman, J.L., Sayers, J., Chapman, C., and Pellet-Many, C. (2020). Emerging Roles for Neuropilin-2 in Cardiovascular Disease. Int J Mol Sci *21*.

Hartikainen, J., Hassinen, I., Hedman, A., Kivela, A., Saraste, A., Knuuti, J., Husso, M., Mussalo, H., Hedman, M., Rissanen, T.T., et al. (2017). Adenoviral intramyocardial VEGF-DDeltaNDeltaC gene transfer increases myocardial perfusion reserve in refractory angina patients: a phase I/IIa study with 1-year follow-up. Eur Heart J *38*, 2547-2555.

Hawley, J.A., Hargreaves, M., Joyner, M.J., and Zierath, J.R. (2014). Integrative biology of exercise. Cell *159*, 738-749.

Heinolainen, K., Karaman, S., D'Amico, G., Tammela, T., Sormunen, R., Eklund, L., Alitalo, K., and Zarkada, G. (2017). VEGFR3 Modulates Vascular Permeability by Controlling VEGF/VEGFR2 Signaling. Circ Res *120*, 1414-1425.

Hemanthakumar, K.A., and Kivela, R. (2020). Angiogenesis and angiocrines regulating heart growth. Vasc Biol *2*, R93-R104.

Heusch, G., Libby, P., Gersh, B., Yellon, D., Bohm, M., Lopaschuk, G., and Opie, L. (2014). Cardiovascular remodelling in coronary artery disease and heart failure. Lancet *383*, 1933-1943.

Hiratsuka, S., Kataoka, Y., Nakao, K., Nakamura, K., Morikawa, S., Tanaka, S., Katsuki, M., Maru, Y., and Shibuya, M. (2005). Vascular endothelial growth factor A (VEGF-A) is involved in guidance of VEGF receptor-positive cells to the anterior portion of early embryos. Mol Cell Biol *25*, 355-363.

Hiratsuka, S., Maru, Y., Okada, A., Seiki, M., Noda, T., and Shibuya, M. (2001). Involvement of Flt-1 tyrosine kinase (vascular endothelial growth factor receptor-1) in pathological angiogenesis. Cancer Res *61*, 1207-1213.

Hiratsuka, S., Minowa, O., Kuno, J., Noda, T., and Shibuya, M. (1998). Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. Proc Natl Acad Sci U S A *95*, 9349-9354.

Ho, V.C., Duan, L.J., Cronin, C., Liang, B.T., and Fong, G.H. (2012). Elevated vascular endothelial growth factor receptor-2 abundance contributes to increased angiogenesis in vascular endothelial growth factor receptor-1-deficient mice. Circulation *126*, 741-752.

Hohensinner, P.J., Kaun, C., Buchberger, E., Ebenbauer, B., Demyanets, S., Huk, I., Eppel, W., Maurer, G., Huber, K., and Wojta, J. (2016). Age intrinsic loss of telomere protection via TRF1 reduction in endothelial cells. Biochim Biophys Acta *1863*, 360-367.

Hooper, A.T., Butler, J.M., Nolan, D.J., Kranz, A., Iida, K., Kobayashi, M., Kopp, H.G., Shido, K., Petit, I., Yanger, K., et al. (2009). Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. Cell stem cell *4*, 263-274.

Hudlicka, O., Brown, M., and Egginton, S. (1992). Angiogenesis in skeletal and cardiac muscle. Physiol Rev *72*, 369-417.

Hulshoff, M.S., Xu, X., Krenning, G., and Zeisberg, E.M. (2018). Epigenetic Regulation of Endothelial-to-Mesenchymal Transition in Chronic Heart Disease. Arterioscler Thromb Vasc Biol *38*, 1986-1996.

Huusko, J., Lottonen, L., Merentie, M., Gurzeler, E., Anisimov, A., Miyanohara, A., Alitalo, K., Tavi, P., and Yla-Herttuala, S. (2012). AAV9-mediated VEGF-B gene transfer improves systolic function in progressive left ventricular hypertrophy. Molecular therapy : the journal of the American Society of Gene Therapy *20*, 2212-2221.

Ishida, J., Hashimoto, T., Hashimoto, Y., Nishiwaki, S., Iguchi, T., Harada, S., Sugaya, T., Matsuzaki, H., Yamamoto, R., Shiota, N., et al. (2004). Regulatory roles for APJ, a seven-transmembrane receptor related to angiotensin-type 1 receptor in blood pressure in vivo. The Journal of biological chemistry *279*, 26274-26279.

Islam, S., Boström, K.I., Di Carlo, D., Simmons, C.A., Tintut, Y., Yao, Y., and Hsu, J.J. (2021). The Mechanobiology of Endothelial-to-Mesenchymal Transition in Cardiovascular Disease. Frontiers in Physiology *12*.

Ito, S., and Nagata, K. (2019). Roles of the endoplasmic reticulum-resident, collagen-specific molecular chaperone Hsp47 in vertebrate cells and human disease. J Biol Chem *294*, 2133-2141.

Jaba, I.M., Zhuang, Z.W., Li, N., Jiang, Y., Martin, K.A., Sinusas, A.J., Papademetris, X., Simons, M., Sessa, W.C., Young, L.H., et al. (2013). NO triggers RGS4 degradation to coordinate angiogenesis and cardiomyocyte growth. J Clin Invest *123*, 1718-1731.

Jaffe, E.A. (1987). Cell biology of endothelial cells. Hum Pathol *18*, 234-239.

James, D., and Rafii, S. (2014). Maladapted endothelial cells flip the mesenchymal switch. Sci Transl Med *6*, 227fs212.

Jia, G., Aroor, A.R., Jia, C., and Sowers, J.R. (2019). Endothelial cell senescence in aging-related vascular dysfunction. Biochim Biophys Acta Mol Basis Dis *1865*, 1802-1809.

Kallio, M.A., Tuimala, J.T., Hupponen, T., Klemela, P., Gentile, M., Scheinin, I., Koski, M., Kaki, J., and Korpelainen, E.I. (2011). Chipster: user-friendly analysis software for microarray and other high-throughput data. BMC Genomics *12*, 507.

Kalucka, J., de Rooij, L., Goveia, J., Rohlenova, K., Dumas, S.J., Meta, E., Conchinha, N.V., Taverna, F., Teuwen, L.A., Veys, K., et al. (2020). Single-Cell Transcriptome Atlas of Murine Endothelial Cells. Cell *180*, 764-779 e720.

Kamo, T., Akazawa, H., and Komuro, I. (2015). Cardiac nonmyocytes in the hub of cardiac hypertrophy. Circ Res *117*, 89-98.
Karkkainen, M.J., Haiko, P., Sainio, K., Partanen, J., Taipale, J., Petrova, T.V., Jeltsch, M., Jackson, D.G., Talikka, M., Rauvala, H., et al. (2004). Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. Nature immunology *5*, 74-80.

Karpanen, T., Bry, M., Ollila, H.M., Seppanen-Laakso, T., Liimatta, E., Leskinen, H., Kivela, R., Helkamaa, T., Merentie, M., Jeltsch, M., et al. (2008). Overexpression of vascular endothelial growth factor-B in mouse heart alters cardiac lipid metabolism and induces myocardial hypertrophy. Circ Res *103*, 1018-1026.

Karpanen, T., Heckman, C.A., Keskitalo, S., Jeltsch, M., Ollila, H., Neufeld, G., Tamagnone, L., and Alitalo, K. (2006). Functional interaction of VEGF-C and VEGF-D with neuropilin receptors. FASEB J *20*, 1462-1472.

Kato, T., Sekiguchi, A., Sagara, K., Tanabe, H., Takamura, M., Kaneko, S., Aizawa, T., Fu, L.T., and Yamashita, T. (2017). Endothelial-mesenchymal transition in human atrial fibrillation. J Cardiol *69*, 706-711.

Kendall, R.L., and Thomas, K.A. (1993). Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci U S A *90*, 10705-10709.

Kim, K.J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H.S., and Ferrara, N. (1993). Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature *362*, 841-844.

Kitsukawa, T., Shimono, A., Kawakami, A., Kondoh, H., and Fujisawa, H. (1995). Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs. Development *121*, 4309-4318.

Kivela, R., Bry, M., Robciuc, M.R., Rasanen, M., Taavitsainen, M., Silvola, J.M., Saraste, A., Hulmi, J.J., Anisimov, A., Mayranpaa, M.I., et al. (2014). VEGF-B-induced vascular growth leads to metabolic reprogramming and ischemia resistance in the heart. EMBO molecular medicine *6*, 307-321.

Koch, S., and Claesson-Welsh, L. (2012). Signal transduction by vascular endothelial growth factor receptors. Cold Spring Harb Perspect Med *2*, a006502.

Kolodkin, A.L., Levengood, D.V., Rowe, E.G., Tai, Y.T., Giger, R.J., and Ginty, D.D. (1997). Neuropilin is a semaphorin III receptor. Cell *90*, 753-762.

Koponen, S., Kokki, E., Kinnunen, K., and Yla-Herttuala, S. (2021). Viral-Vector-Delivered Anti-Angiogenic Therapies to the Eye. Pharmaceutics *13*.

Kovacic, J.C., Dimmeler, S., Harvey, R.P., Finkel, T., Aikawa, E., Krenning, G., and Baker, A.H. (2019). Endothelial to Mesenchymal Transition in Cardiovascular Disease: JACC State-of-the-Art Review. J Am Coll Cardiol *73*, 190-209.

Kukk, E., Lymboussaki, A., Taira, S., Kaipainen, A., Jeltsch, M., Joukov, V., and Alitalo, K. (1996). VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. Development *122*, 3829-3837.

Lahteenvuo, J., Hatinen, O.P., Kuivanen, A., Huusko, J., Paananen, J., Lahteenvuo, M., Nurro, J., Hedman, M., Hartikainen, J., Laham-Karam, N., et al. (2020). Susceptibility to Cardiac Arrhythmias and Sympathetic Nerve Growth in VEGF-B Overexpressing Myocardium. Mol Ther *28*, 1731-1740.

Le Bras, B., Barallobre, M.J., Homman-Ludiye, J., Ny, A., Wyns, S., Tammela, T., Haiko, P., Karkkainen, M.J., Yuan, L., Muriel, M.P., et al. (2006). VEGF-C is a trophic factor for neural progenitors in the vertebrate embryonic brain. Nat Neurosci *9*, 340-348.

Lee, S., Chen, T.T., Barber, C.L., Jordan, M.C., Murdock, J., Desai, S., Ferrara, N., Nagy, A., Roos, K.P., and Iruela-Arispe, M.L. (2007). Autocrine VEGF signaling is required for vascular homeostasis. Cell *130*, 691-703.

Leikas, A.J., Laham-Karam, N., Agtereek, E., Peltonen, H.M., Selander, T., Korpisalo, P., Holappa, L., Hartikainen, J.E.K., Heikura, T., and Yla-Herttuala, S. (2021). Efficacy and Safety of Clinical-Grade Human Vascular Endothelial Growth Factor-D(DeltaNDeltaC) Gene Therapy Containing Residual Replication-Competent Adenoviruses. Hum Gene Ther.

Li, Y., Lui, K.O., and Zhou, B. (2018). Reassessing endothelial-to-mesenchymal transition in cardiovascular diseases. Nat Rev Cardiol *15*, 445-456.

Liu, X., De la Cruz, E., Gu, X., Balint, L., Oxendine-Burns, M., Terrones, T., Ma, W., Kuo, H.H., Lantz, C., Bansal, T., et al. (2020). Lymphoangiocrine signals promote cardiac growth and repair. Nature *588*, 705-711.

Lois, C., Hong, E.J., Pease, S., Brown, E.J., and Baltimore, D. (2002). Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science *295*, 868-872.

Lopez-Otin, C., Blasco, M.A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The hallmarks of aging. Cell *153*, 1194-1217.

Maddaluno, L., Rudini, N., Cuttano, R., Bravi, L., Giampietro, C., Corada, M., Ferrarini, L., Orsenigo, F., Papa, E., Boulday, G., et al. (2013). EndMT contributes to the onset and progression of cerebral cavernous malformations. Nature *498*, 492-496.

Maes, C., Carmeliet, P., Moermans, K., Stockmans, I., Smets, N., Collen, D., Bouillon, R., and Carmeliet, G. (2002). Impaired angiogenesis and endochondral bone formation in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. Mech Dev *111*, 61-73.

Magenta, A., Cencioni, C., Fasanaro, P., Zaccagnini, G., Greco, S., Sarra-Ferraris, G., Antonini, A., Martelli, F., and Capogrossi, M.C. (2011). miR-200c is upregulated by oxidative stress and induces endothelial cell apoptosis and senescence via ZEB1 inhibition. Cell Death Differ *18*, 1628-1639.

Maglione, D., Guerriero, V., Viglietto, G., Delli-Bovi, P., and Persico, M.G. (1991). Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. Proc Natl Acad Sci U S A *88*, 9267-9271.

Maglione, D., Guerriero, V., Viglietto, G., Ferraro, M.G., Aprelikova, O., Alitalo, K., Del Vecchio, S., Lei, K.J., Chou, J.Y., and Persico, M.G. (1993). Two alternative mRNAs coding for the angiogenic factor, placenta growth factor (PlGF), are transcribed from a single gene of chromosome 14. Oncogene *8*, 925-931.

Maillet, M., van Berlo, J.H., and Molkentin, J.D. (2013). Molecular basis of physiological heart growth: fundamental concepts and new players. Nature reviews. Molecular cell biology *14*, 38-48.

Maiorana, A., O'Driscoll, G., Cheetham, C., Dembo, L., Stanton, K., Goodman, C., Taylor, R., and Green, D. (2001). The effect of combined aerobic and resistance exercise training on vascular function in type 2 diabetes. J Am Coll Cardiol *38*, 860-866.

Makinen, T., Olofsson, B., Karpanen, T., Hellman, U., Soker, S., Klagsbrun, M., Eriksson, U., and Alitalo, K. (1999). Differential binding of vascular endothelial growth factor B splice and proteolytic isoforms to neuropilin-1. The Journal of biological chemistry *274*, 21217-21222.

McColl, B.K., Baldwin, M.E., Roufail, S., Freeman, C., Moritz, R.L., Simpson, R.J., Alitalo, K., Stacker, S.A., and Achen, M.G. (2003). Plasmin activates the lymphangiogenic growth factors VEGF-C and VEGF-D. J Exp Med *198*, 863-868.

McLaren, J., Prentice, A., Charnock-Jones, D.S., Millican, S.A., Muller, K.H., Sharkey, A.M., and Smith, S.K. (1996). Vascular endothelial growth factor is produced by peritoneal fluid macrophages in endometriosis and is regulated by ovarian steroids. J Clin Invest *98*, 482-489.

Mendis S, P.P., Norrving B (2011). Global Atlas on Cardiovascular Disease Prevention and Control. (Geneva: World Health Organization).

Meyer, R.D., Mohammadi, M., and Rahimi, N. (2006). A single amino acid substitution in the activation loop defines the decoy characteristic of VEGFR-1/FLT-1. J Biol Chem *281*, 867-875.

Naumenko, N., Huusko, J., Tuomainen, T., Koivumaki, J.T., Merentie, M., Gurzeler, E., Alitalo, K., Kivela, R., Yla-Herttuala, S., and Tavi, P. (2017). Vascular Endothelial Growth Factor-B Induces a Distinct Electrophysiological Phenotype in Mouse Heart. Front Physiol *8*, 373.

Nesmith, J.E., Chappell, J.C., Cluceru, J.G., and Bautch, V.L. (2017). Blood vessel anastomosis is spatially regulated by Flt1 during angiogenesis. Development *144*, 889-896.

Nolan, D.J., Ginsberg, M., Israely, E., Palikuqi, B., Poulos, M.G., James, D., Ding, B.S., Schachterle, W., Liu, Y., Rosenwaks, Z., et al. (2013). Molecular signatures of tissue-specific microvascular endothelial cell heterogeneity in organ maintenance and regeneration. Developmental cell *26*, 204-219.

Nurro, J., Halonen, P.J., Kuivanen, A., Tarkia, M., Saraste, A., Honkonen, K., Lahteenvuo, J., Rissanen, T.T., Knuuti, J., and Yla-Herttuala, S. (2016). AdVEGF-B186 and AdVEGF-DDeltaNDeltaC induce angiogenesis and increase perfusion in porcine myocardium. Heart *102*, 1716-1720.

OECD (2015). Cardiovascular Disease and Diabetes: Policies for Better Health and Quality of Care.

Oka, T., Akazawa, H., Naito, A.T., and Komuro, I. (2014). Angiogenesis and cardiac hypertrophy: maintenance of cardiac function and causative roles in heart failure. Circ Res *114*, 565-571.

Okabe, K., Kobayashi, S., Yamada, T., Kurihara, T., Tai-Nagara, I., Miyamoto, T., Mukouyama, Y.S., Sato, T.N., Suda, T., Ema, M., et al. (2014). Neurons limit angiogenesis by titrating VEGF in retina. Cell *159*, 584-596.

Olofsson, B., Korpelainen, E., Pepper, M.S., Mandriota, S.J., Aase, K., Kumar, V., Gunji, Y., Jeltsch, M.M., Shibuya, M., Alitalo, K., et al. (1998). Vascular endothelial growth factor B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells. Proc Natl Acad Sci U S A *95*, 11709-11714.

Olofsson, B., Pajusola, K., Kaipainen, A., von Euler, G., Joukov, V., Saksela, O., Orpana, A., Pettersson, R.F., Alitalo, K., and Eriksson, U. (1996). Vascular endothelial growth factor B, a novel growth factor for endothelial cells. Proc Natl Acad Sci U S A *93*, 2576-2581.

Olsson, A.K., Dimberg, A., Kreuger, J., and Claesson-Welsh, L. (2006). VEGF receptor signalling - in control of vascular function. Nature reviews. Molecular cell biology *7*, 359-371.

Orlandini, M., Spreafico, A., Bardelli, M., Rocchigiani, M., Salameh, A., Nucciotti, S., Capperucci, C., Frediani, B., and Oliviero, S. (2006). Vascular endothelial growth factor-D activates VEGFR-3 expressed in osteoblasts inducing their differentiation. J Biol Chem *281*, 17961-17967.

Paradies, P., Carlucci, L., Woitek, F., Staffieri, F., Lacitignola, L., Ceci, L., Romano, D., Sasanelli, M., Zentilin, L., Giacca, M., et al. (2019). Intracoronary Gene Delivery of the Cytoprotective Factor Vascular Endothelial Growth Factor-B167 in Canine Patients with Dilated Cardiomyopathy: A Short-Term Feasibility Study. Vet Sci *6*.

Pasquier, J., Ghiabi, P., Chouchane, L., Razzouk, K., Rafii, S., and Rafii, A. (2020). Angiocrine endothelium: from physiology to cancer. J Transl Med *18*, 52.

Pepe, M., Mamdani, M., Zentilin, L., Csiszar, A., Qanud, K., Zacchigna, S., Ungvari, Z., Puligadda, U., Moimas, S., Xu, X., et al. (2010). Intramyocardial VEGF-B167 gene delivery delays the progression towards congestive failure in dogs with pacing-induced dilated cardiomyopathy. Circ Res *106*, 1893-1903.

Pertovaara, L., Kaipainen, A., Mustonen, T., Orpana, A., Ferrara, N., Saksela, O., and Alitalo, K. (1994). Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. J Biol Chem *269*, 6271-6274.

Peterson, A.L., and Waterhouse, A.L. (2016). (1)H NMR: A Novel Approach To Determining the Thermodynamic Properties of Acetaldehyde Condensation Reactions with Glycerol, (+)-Catechin, and Glutathione in Model Wine. J Agric Food Chem *64*, 6869-6878.

Pinto, A.R., Ilinykh, A., Ivey, M.J., Kuwabara, J.T., D'Antoni, M.L., Debuque, R., Chandran, A., Wang, L., Arora, K., Rosenthal, N.A., et al. (2016). Revisiting Cardiac Cellular Composition. Circ Res *118*, 400-409.

Poesen, K., Lambrechts, D., Van Damme, P., Dhondt, J., Bender, F., Frank, N., Bogaert, E., Claes, B., Heylen, L., Verheyen, A., et al. (2008). Novel role for vascular endothelial growth factor (VEGF) receptor-1 and its ligand VEGF-B in motor neuron degeneration. J Neurosci *28*, 10451-10459.

Pugh, C.W., and Ratcliffe, P.J. (2003). Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med *9*, 677-684.

Radu, M., and Chernoff, J. (2013). An in vivo assay to test blood vessel permeability. J Vis Exp, e50062.

Rafii, S., Butler, J.M., and Ding, B.S. (2016). Angiocrine functions of organ-specific endothelial cells. Nature *529*, 316-325.

Rasanen, M., Degerman, J., Nissinen, T.A., Miinalainen, I., Kerkela, R., Siltanen, A., Backman, J.T., Mervaala, E., Hulmi, J.J., Kivela, R., et al. (2016). VEGF-B gene therapy inhibits doxorubicin-induced cardiotoxicity by endothelial protection. Proc Natl Acad Sci U S A *113*, 13144-13149.

Ribatti, D. (2008). The discovery of the placental growth factor and its role in angiogenesis: a historical review. Angiogenesis *11*, 215-221.

Rieder, F., Kessler, S.P., West, G.A., Bhilocha, S., de la Motte, C., Sadler, T.M., Gopalan, B., Stylianou, E., and Fiocchi, C. (2011). Inflammation-induced endothelial-to-mesenchymal transition: a novel mechanism of intestinal fibrosis. Am J Pathol *179*, 2660-2673.

Risau, W., and Flamme, I. (1995). Vasculogenesis. Annu Rev Cell Dev Biol *11*, 73-91.

Rissanen, T.T., and Yla-Herttuala, S. (2007). Current status of cardiovascular gene therapy. Mol Ther *15*, 1233-1247.

Robciuc, M.R., Kivela, R., Williams, I.M., de Boer, J.F., van Dijk, T.H., Elamaa, H., Tigistu-Sahle, F., Molotkov, D., Leppanen, V.M., Kakela, R., et al. (2016). VEGFB/VEGFR1-Induced Expansion of Adipose Vasculature Counteracts Obesity and Related Metabolic Complications. Cell Metab *23*, 712-724.

Rossman, M.J., Kaplon, R.E., Hill, S.D., McNamara, M.N., Santos-Parker, J.R., Pierce, G.L., Seals, D.R., and Donato, A.J. (2017). Endothelial cell senescence with aging in healthy humans: prevention by habitual exercise and relation to vascular endothelial function. Am J Physiol Heart Circ Physiol *313*, H890-H895.

Sabayan, B., Westendorp, R.G., Grond, J., Stott, D.J., Sattar, N., van Osch, M.J., van Buchem, M.A., and de Craen, A.J. (2014). Markers of endothelial dysfunction and cerebral blood flow in older adults. Neurobiol Aging *35*, 373-377.

Sadler, T.W., 2006. Langman's medical embryology, 10th ed. Lippincott Williams & Wilkins, Philadelphia.

Sanchez-Duffhues, G., Garcia de Vinuesa, A., and Ten Dijke, P. (2018). Endothelial-to-mesenchymal transition in cardiovascular diseases: Developmental signaling pathways gone awry. Dev Dyn *247*, 492-508.

Santamaria, P.G., Moreno-Bueno, G., Portillo, F., and Cano, A. (2017). EMT: Present and future in clinical oncology. Mol Oncol *11*, 718-738.

Sawano, A., Takahashi, T., Yamaguchi, S., Aonuma, M., and Shibuya, M. (1996). Flt-1 but not KDR/Flk-1 tyrosine kinase is a receptor for placenta growth factor, which is related to vascular endothelial growth factor. Cell Growth Differ *7*, 213-221.

Schmeisser, A., Christoph, M., Augstein, A., Marquetant, R., Kasper, M., Braun-Dullaeus, R.C., and Strasser, R.H. (2006). Apoptosis of human macrophages by Flt-4 signaling: implications for atherosclerotic plaque pathology. Cardiovasc Res *71*, 774-784.

Secker, G.A., and Harvey, N.L. (2015). VEGFR signaling during lymphatic vascular development: From progenitor cells to functional vessels. Dev Dyn *244*, 323-331.

Senger, D.R., Galli, S.J., Dvorak, A.M., Perruzzi, C.A., Harvey, V.S., and Dvorak, H.F. (1983). Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science *219*, 983-985.

Serpi, R., Tolonen, A.M., Huusko, J., Rysa, J., Tenhunen, O., Yla-Herttuala, S., and Ruskoaho, H. (2011). Vascular endothelial growth factor-B gene transfer prevents angiotensin II-induced diastolic dysfunction via proliferation and capillary dilatation in rats. Cardiovascular research *89*, 204-213.

Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertsenstein, M., Wu, X.F., Breitman, M.L., and Schuh, A.C. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature *376*, 62-66.

Shibuya, M. (2013). Vascular endothelial growth factor and its receptor system: physiological functions in angiogenesis and pathological roles in various diseases. J Biochem *153*, 13-19.

Shinkai, A., Ito, M., Anazawa, H., Yamaguchi, S., Shitara, K., and Shibuya, M. (1998). Mapping of the sites involved in ligand association and dissociation at the extracellular domain of the kinase insert domain-containing receptor for vascular endothelial growth factor. J Biol Chem *273*, 31283-31288.

Simons, M., Gordon, E., and Claesson-Welsh, L. (2016). Mechanisms and regulation of endothelial VEGF receptor signalling. Nat Rev Mol Cell Biol *17*, 611-625.

Singhal, M., and Augustin, H.G. (2020). Beyond Angiogenesis: Exploiting Angiocrine Factors to Restrict Tumor Progression and Metastasis. Cancer Res *80*, 659-662.

Stalmans, I., Ng, Y.S., Rohan, R., Fruttiger, M., Bouche, A., Yuce, A., Fujisawa, H., Hermans, B., Shani, M., Jansen, S., et al. (2002). Arteriolar and venular patterning in retinas of mice selectively expressing VEGF isoforms. J Clin Invest *109*, 327-336.

Sun, Y., Jin, K., Childs, J.T., Xie, L., Mao, X.O., and Greenberg, D.A. (2004). Increased severity of cerebral ischemic injury in vascular endothelial growth factor-B-deficient mice. J Cereb Blood Flow Metab *24*, 1146-1152.

Sun, Y., Jin, K., Childs, J.T., Xie, L., Mao, X.O., and Greenberg, D.A. (2006). Vascular endothelial growth factor-B (VEGFB) stimulates neurogenesis: evidence from knockout mice and growth factor administration. Dev Biol *289*, 329-335.

Tabula Muris, C., Overall, c., Logistical, c., Organ, c., processing, Library, p., sequencing, Computational data, a., Cell type, a., Writing, g., et al. (2018). Single-cell transcriptomics of 20 mouse organs creates a Tabula Muris. Nature *562*, 367-372.

Taichman, N.S., Young, S., Cruchley, A.T., Taylor, P., and Paleolog, E. (1997). Human neutrophils secrete vascular endothelial growth factor. J Leukoc Biol *62*, 397-400.

Takahashi, H., and Shibuya, M. (2005). The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. Clin Sci (Lond) *109*, 227-241.

Takashima, S., Kitakaze, M., Asakura, M., Asanuma, H., Sanada, S., Tashiro, F., Niwa, H., Miyazaki Ji, J., Hirota, S., Kitamura, Y., et al. (2002). Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. Proc Natl Acad Sci U S A *99*, 3657-3662.

Talman, V., and Kivela, R. (2018). Cardiomyocyte-Endothelial Cell Interactions in Cardiac Remodeling and Regeneration. Front Cardiovasc Med *5*, 101.

Tammela, T., and Alitalo, K. (2010). Lymphangiogenesis: Molecular mechanisms and future promise. Cell *140*, 460-476.

Tammela, T., Zarkada, G., Wallgard, E., Murtomaki, A., Suchting, S., Wirzenius, M., Waltari, M., Hellstrom, M., Schomber, T., Peltonen, R., et al. (2008). Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. Nature *454*, 656-660.

Tang, J., Zhang, H., He, L., Huang, X., Li, Y., Pu, W., Yu, W., Zhang, L., Cai, D., Lui, K.O., et al. (2018). Genetic Fate Mapping Defines the Vascular Potential of Endocardial Cells in the Adult Heart. Circ Res *122*, 984-993.

Tian, X., Hu, T., Zhang, H., He, L., Huang, X., Liu, Q., Yu, W., He, L., Yang, Z., Zhang, Z., et al. (2013). Subepicardial endothelial cells invade the embryonic ventricle wall to form coronary arteries. Cell Res *23*, 1075-1090.

Tirziu, D., Chorianopoulos, E., Moodie, K.L., Palac, R.T., Zhuang, Z.W., Tjwa, M., Roncal, C., Eriksson, U., Fu, Q., Elfenbein, A., et al. (2007). Myocardial hypertrophy in the absence of external stimuli is induced by angiogenesis in mice. The Journal of clinical investigation *117*, 3188-3197.

Tirziu, D., Giordano, F.J., and Simons, M. (2010). Cell communications in the heart. Circulation *122*, 928-937.

Tombor, L.S., John, D., Glaser, S.F., Luxan, G., Forte, E., Furtado, M., Rosenthal, N., Baumgarten, N., Schulz, M.H., Wittig, J., et al. (2021). Single cell sequencing reveals endothelial plasticity with transient mesenchymal activation after myocardial infarction. Nature communications *12*, 681.

Ungvari, Z., Tarantini, S., Kiss, T., Wren, J.D., Giles, C.B., Griffin, C.T., Murfee, W.L., Pacher, P., and Csiszar, A. (2018). Endothelial dysfunction and angiogenesis impairment in the ageing vasculature. Nat Rev Cardiol *15*, 555-565.

Uryga, A.K., and Bennett, M.R. (2016). Ageing induced vascular smooth muscle cell senescence in atherosclerosis. J Physiol *594*, 2115-2124.

van Berlo, J.H., Maillet, M., and Molkentin, J.D. (2013). Signaling effectors underlying pathologic growth and remodeling of the heart. The Journal of clinical investigation *123*, 37-45.

Veikkola, T., Jussila, L., Makinen, T., Karpanen, T., Jeltsch, M., Petrova, T.V., Kubo, H., Thurston, G., McDonald, D.M., Achen, M.G., et al. (2001). Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. EMBO J *20*, 1223-1231.

Vousden, K.H., and Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53. Cell *137*, 413-431.

Vujic, A., Lerchenmuller, C., Wu, T.D., Guillermier, C., Rabolli, C.P., Gonzalez, E., Senyo, S.E., Liu, X., Guerquin-Kern, J.L., Steinhauser, M.L., et al. (2018). Exercise induces new cardiomyocyte generation in the adult mammalian heart. Nat Commun *9*, 1659.

Walsh, K., and Shiojima, I. (2007). Cardiac growth and angiogenesis coordinated by intertissue interactions. The Journal of clinical investigation *117*, 3176-3179.

Wessels, A., and Sedmera, D. (2003). Developmental anatomy of the heart: a tale of mice and man. Physiol Genomics *15*, 165-176.

White, F.C., Bloor, C.M., McKirnan, M.D., and Carroll, S.M. (1998). Exercise training in swine promotes growth of arteriolar bed and capillary angiogenesis in heart. J Appl Physiol (1985) *85*, 1160-1168.

Woitek, F., Zentilin, L., Hoffman, N.E., Powers, J.C., Ottiger, I., Parikh, S., Kulczycki, A.M., Hurst, M., Ring, N., Wang, T., et al. (2015). Intracoronary Cytoprotective Gene Therapy: A Study of VEGF-B167 in a Pre-Clinical Animal Model of Dilated Cardiomyopathy. J Am Coll Cardiol *66*, 139-153.

Wu, D., and Prives, C. (2018). Relevance of the p53-MDM2 axis to aging. Cell Death Differ *25*, 169-179.

Xin, M., Olson, E.N., and Bassel-Duby, R. (2013). Mending broken hearts: cardiac development as a basis for adult heart regeneration and repair. Nat Rev Mol Cell Biol *14*, 529-541.

Xiong, J., Kawagishi, H., Yan, Y., Liu, J., Wells, Q.S., Edmunds, L.R., Fergusson, M.M., Yu, Z.X., Rovira, II, Brittain, E.L., et al. (2018). A Metabolic Basis for Endothelial-to-Mesenchymal Transition. Mol Cell *69*, 689-698 e687.

Xu, Y., Yuan, L., Mak, J., Pardanaud, L., Caunt, M., Kasman, I., Larrivee, B., Del Toro, R., Suchting, S., Medvinsky, A., et al. (2010). Neuropilin-2 mediates VEGF-C-induced lymphatic sprouting together with VEGFR3. J Cell Biol *188*, 115-130.

Zacchigna, S., Zentilin, L., and Giacca, M. (2014). Adeno-associated virus vectors as therapeutic and investigational tools in the cardiovascular system. Circ Res *114*, 1827-1846.

Zarkada, G., Heinolainen, K., Makinen, T., Kubota, Y., and Alitalo, K. (2015). VEGFR3 does not sustain retinal angiogenesis without VEGFR2. Proc Natl Acad Sci U S A *112*, 761-766.

Zeisberg, E.M., Potenta, S.E., Sugimoto, H., Zeisberg, M., and Kalluri, R. (2008). Fibroblasts in kidney fibrosis emerge via endothelial-to-mesenchymal transition. J Am Soc Nephrol *19*, 2282-2287.

Zeisberg, E.M., Tarnavski, O., Zeisberg, M., Dorfman, A.L., McMullen, J.R., Gustafsson, E., Chandraker, A., Yuan, X., Pu, W.T., Roberts, A.B., et al. (2007). Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. Nat Med *13*, 952-961.

Zentilin, L., Puligadda, U., Lionetti, V., Zacchigna, S., Collesi, C., Pattarini, L., Ruozi, G., Camporesi, S., Sinagra, G., Pepe, M., et al. (2010). Cardiomyocyte VEGFR-1 activation by VEGF-B induces compensatory hypertrophy and preserves cardiac function after myocardial infarction. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *24*, 1467-1478.

Zhang, L., Hoffman, J.A., and Ruoslahti, E. (2005). Molecular profiling of heart endothelial cells. Circulation *112*, 1601-1611.

Zhou, P., and Pu, W.T. (2016). Recounting Cardiac Cellular Composition. Circ Res *118*, 368-370.